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INTERNATIONAL APPLICATION NO.
PCT/US00/29231 ✓INTERNATIONAL FILING DATES
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TITLE OF INVENTION VACCINE COMPOSITIONS ✓

APPLICANT(S)
FOR DO/EO/US

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Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to promptly begin national examination procedures (35 U.S.C. 371 (f)).
4. ☐ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c)(2))
 - a. ☐ is attached hereto (required only if not transmitted by the International Bureau).
 - b. ☐ has been communicated by the International Bureau.
 - c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371 (c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ have been communicated by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98. w/PTO-1449, 2 references
12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 & 3.31 is included.
13. ☐ A FIRST preliminary amendment.
- ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:

-Copy of International Application as published
 -Ten (10) Sheets Drawings
 -Sequence Listing
 -Statement accompanying Sequence Listing
 -Diskette Containing Sequence Listing

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Page 1 of 2

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VACCINE COMPOSITIONS

TECHNICAL FIELD

This invention relates to avirulent or attenuated microbes, their method of preparation, and their use in vaccines.

5 BACKGROUND ART

Infectious disease remains the largest cause of mortality in the world. A significant proportion of infectious disease associated morbidity and mortality results from bacterial pathogens. One widely applied technique used in controlling the spread and severity of bacterial infection is vaccination. Several notable vaccine
10 examples prevent a number of lethal microbial diseases, the DPT vaccine protects against diphtheria, pertusis and tetanus. The BCG or bacillus Calmette-Guerin vaccine is an example of an attenuated strain which is used worldwide to control the spread of tuberculosis (TB). A central issue in the development of safe and effective bacterial vaccines is the identification of protective antigens or attenuated strains of
15 bacteria which can promote the development of an immune response in the host yet in vaccine composition fail to cause morbidity or mortality in the host. A variety of methods can be employed to identify vaccine compositions including the production of attenuated or killed vaccine strains. Often these strains are in single genes which have been shown to play a role in virulence of the organism. Vaccine compositions
20 can include a live or fixed bacterial preparation of the mutant strain, the fixed protein, a fusion protein made of the virulence gene product and a suitable carrier and more recently the DNA encoding the protein.

Attenuated strains offer the potential of presenting a nearly intact complement of pathogen associated antigens to immune system. Furthermore these
25 antigens are likely to be presented in a bacterial context that mirrors that seen during early stages of infection by a virulent strain. This is evidenced by the ongoing practice of using live attenuated strains in the extensive vaccination of both human and livestock. For example BCG for tuberculosis and strain 19 against bovine brucellosis and Sterne's spore vaccine against anthrax in cattle.

30 The use of live vaccines can present developmental obstacles including the retention of unacceptable levels of virulence, the risk of reversion to

virulence during culture or *in vivo* and lack of efficacy. The ability to create more effective live or attenuated vaccines is in part dependent upon the ability to control and restrict the expression of virulence determinants so as to create vaccine strains that are protective and safe.

5 Bacteria respond to nutritional stress by the coordinated expression of different genes. This facilitates their survival in different environments. Among these differentially regulated genes are the genes responsible for the expression of virulence determinants. The selective expression of these genes in a sensitive or susceptible host allows for the establishment and maintenance of infection or disease.

10 Virulence include genes which encode toxins, colonization factors and genes required for siderophores production or other factors that promote this process.

 The expression of virulence genes in bacteria therefore enable the organism to invade, colonize and initiate an infection in humans and/or animals, however, these genes are not necessarily expressed constantly (constitutively). That

15 is, the bacterium is not always orchestrating gene expression patterns to maximize "infectious" potential. In many circumstances, the expression of virulence genes is controlled by regulatory circuitry which include repressor proteins and a corresponding operon or operator. One class of repressors which is activated upon binding to or forming a complex with a transition metal ion such as iron, zinc or

20 manganese is thought to control the expression of a subset of genes in a number of Gram positive organisms. When such repressors are activated and associated with virulence gene expression in pathogens, they bind the operator sites thereby preventing production of virulence determinants.

 Virulence determinants are most often expressed when the bacterial

25 pathogen is exposed to environmental stress such nutritional restriction. An iron-poor environment is an example of such a condition. In many eucaryotes such an environment is the norm, insufficient iron is present to maintain the repressor in its active state. In the inactive form, the repressor cannot bind to target operators. As a result, virulence genes are de-repressed and the bacterium is able to initiate, establish,

30 promote or maintain infection.

The expression of these virulence determinants is in many bacterial species co-regulated by metal ions. In most instances the metal co-factor that is involved *in vivo* is iron [but can include zinc, nickel, manganese, cobalts]. In the presence of iron, the repressor is activated and virulence gene expression is halted.

5 This pattern of gene regulation is illustrated by the following example. The bacterium that causes diphtheria produces one of the most potent toxins known to man. The toxin is only produced under conditions of iron deprivation. In the presence of iron, the bacterial repressor (which in this species is known as diphtheria toxin repressor protein, abbreviated "*DtxR*") binds iron and undergoes
10 conformational changes that activate it and allow it to dimerize and bind a specific DNA sequence called the tox operator. The tox operator is a specific DNA sequence found upstream of the gene that produces the diphtheria toxin, thereby preventing its expression. Typically, during infection of a human host the diphtheria bacillus (or other pathogenic/opportunistic bacteria) grows in an environment that rapidly
15 becomes restricted in several key nutrients. Paramount among these essential nutrients is iron, and when iron becomes limiting the diphtheria bacillus begins to produce the toxin. Moreover, the constellation of virulence genes that *DtxR* controls becomes de-repressed and the diphtheria bacillus becomes better adapted to cause an infection. In the case of diphtheria, the toxin kills host cells thereby releasing
20 required nutrients including iron.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 aligns the amino acid sequences of *DtxR*, *IdeR* and *SirR*.

Figure 2 aligns amino acid sequences of various *IdeR*/*SirR* Homologues found in various species of mycobacterium.

25 Figure 3 aligns and compares the amino acid sequence of various homologues of various *DtxR* type repressors, including *DtxR* from *Brevibacterium lactofermentum* (Bl), *DtxR* from *Corynebacterium diphtheriae* (Cd), *IdeR* from *Mycobacterium tuberculosis* (Mt), *M. leprae* [P], *M. smegmatis* [P]; *DesR* from *Streptomyces lividans* (Sl), *M. tuberculosis* *SirR*, *Staphylococcus aureus* (Sa) *SirR*, *S.*
30 *epidermidis* *SirR*, *Enterococcus faecalis* *DtxR* homologue [P]. The *DtxR* homologues from the *Streptococcus gordonii*, *S. mutans*, *S. pneumoniae* and *S. pyogenes*. The

consensus amino acid sequences between these members of the DtxR family of iron-dependent repressors is indicated. *, metal ion coordination residues in the Primary site; #, metal ion coordination residues in the Ancillary site; @, the single amino acid residue that interacts with a base in the binding of DtxR dimers to the *tox* operator.

- 5 AMS has clones of *M. tuberculosis* IdeR, *S. aureus* and *S. epidermidis* SirR, and DtxR homologues from *Enterococcus faecalis*, *S. mutans*, *S. pneumoniae*, and *K. pneumoniae*.

Figure 4: Western blot of cell lysates incubated with polyclonal antibody against DtxR. Lane 1 shows purified DtxR (25.3 kDa). Lanes 3 and 5:
 10 lysates from wild type *M. smegmatis* and *M. tuberculosis*, respectively, expressing native IdeR (25.2 kDa.). Lane 4: lysate from the *M. smegmatis* heterodiploid harboring pNBV1/SAD expressing both DtxR(E175K) and IdeR. The molecular weight masses, determined by size standards, are shown at left.

Figure 5: Virulence comparison of wild type *M. tuberculosis* and
 15 *M. tuberculosis* DtxR(E175K) mutant. Panel A shows the log CFU of the homogenized spleens of mice sacrificed at 4 week intervals. Panel B shows the log CFU of homogenized lungs at 4 week intervals. Each point represents the mean log CFU of 5-6 mice \pm 1 standard deviation (error bars). Asterisks denote statistically significant differences between groups at a given time point.

20 Figure 6: Panel A: a 10 week-old representative colony of wild type *M. tuberculosis* (strain CDC1551) on 7H10 agar. Panel B: a 10 week-old representative colony of *M. tuberculosis* DtxR(E175K) on 7H10 agar.

Figure 7: Alignment of the "iron box" consensus sequence, known DtxR binding sites, and putative *M. tuberculosis* DtxR/IdeR binding sites identified
 25 by an *in silico* genome search. The "consensus sequence" at the top of the figure represents the compilation of the 9 aligned sequences in the figure. The "published consensus" is drawn from the literature. Gene homologues of the downstream ORFs are shown on the right.

Figure 8: Autoradiographs of gel binding assay between DtxR and
 30 putative *M. tuberculosis* DtxR/IdeR binding sites. 100 bp ³²P-end-labeled DNA fragments containing *toxO* (lanes 1 & 2), IB-1 (lanes 3 & 4), IB-2 (lanes 5 & 6), IB-

3 (lanes 7 & 8), IB-4 (lanes 9 & 10), IB-5 (lanes 11 & 12) separated in a non-denaturing 6% polyacrylamide gel. Odd numbered lanes contain DNA only ("unbound"), and even numbered lanes contain DNA pre-incubated with purified DtxR ("bound").

5 Figure 9. Virulence of *S. aureus* is altered in a mouse skin lesion model following 8 days of *in vivo* incubation. 8.0 log CFU of the parent strain MA2181 [RN6390 carrying empty shuttle vector pSPT181] and 7.8 log CFU of the complemented MA2004 strain carrying DtxR E175K were injected sub-cutaneously on day 1 abscess size[mm] was measured each day over 8 days and on the last day
10 the abscess was removed and the number of CFU were determined. CFU [a.] and abscess size [b.] were compared between groups.

SUMMARY OF THE INVENTION

 A first aspect of the present invention is directed to a composition containing a virulent or opportunistic prokaryote in which metal ion-dependent gene
15 regulation confers a growth or an infectious advantage. The prokaryote contains a recombinant DNA molecule comprising a promoter in operable association with a sequence encoding a dominant, metal ion-independent repressor protein or a partially metal ion independent repressor protein, and a carrier. In preferred embodiments, a
20 molecule contains a sequence encoding a metal ion-independent DtxR protein or a partially metal ion-independent DtxR protein. In yet other preferred embodiments, the bacterium is a member of the genus *Mycobacterium*, *Staphylococcus* or *Streptococcus*.

 The second aspect of the present invention is directed to a method of
25 enhancing protective immunity against infection or disease caused by an opportunistic or virulent prokaryote pathogen in which metal or metal ion-dependent gene regulation confers a growth or an infectious advantage. The method entails administering compositions to the animal. In preferred embodiments, the animal is a human. In other preferred embodiments, the compositions are administered
30 prophylactically e.g. prior to the onset of the infection or disease. In yet other

preferred embodiments, the prokaryote contained in the compositions is in a live or killed form.

A relatively aspect directed to is a method of attenuating or reducing the severity of an infection or a disease caused by an opportunistic or virulent prokaryotic pathogen in which metal or metal ion-dependent gene regulation confers a drug or an infectious advantage. The method also entails administering the compositions to the animal, preferably prior to the onset of the infection or disease condition.

Yet another aspect of the present invention is directed to isolated and purified DNA molecules consisting essentially of a sequence encoding a metal or metal ion-independent or a partially metal or metal ion-independent DtxR or homologue thereof. Preferred homologues are IdeR and SirR. In other preferred embodiments, the molecule is placed in an expression cassette or a vector (e.g., a plasmid) so as to be in operable association with a promoter element, especially a constitutive promoter. Vectors containing the DNA molecules and prokaryotes transformed with DNA molecules (including cultures thereof) are also provided.

A further aspect of the present invention is directed to a method for preparing the compositions. The method entails obtaining a DNA molecule encoding a metal ion-independent repressor protein or a partially metal ion-independent repressor protein. The wild type protein, in its native state, is a metal ion-dependent gene regulator and confers upon a virulent or opportunistic prokaryote a growth or an infectious advantage. The DNA molecule is linked to a promoter, preferably a constitutive promoter, and then introduced into such a virulent or opportunist prokaryote. The DNA is expressed in the prokaryote and inhibits metal ion-dependent gene regulation.

The present invention entails the incorporation of an exogenous DNA encoding a dominant, metal ion (hereinafter "metal") independent or partially metal ion independent mutant repressor into an otherwise virulent or opportunistic prokaryote in which metal ion-dependent gene regulation confers a growth or an infectious advantage. Such prokaryotes include gram-positive and gram-negative bacteria. Without intending to be bound by theory, Applicants believe that the

dominance of the metal-independent mutant repressor subverts the normal patterns of gene regulation (under the control of the native, metal-dependent repressor), thereby creating a recombinant prokaryote that is attenuated or avirulent relative to the wild-type prokaryote. That is, the exogenous mutant repressor renders inoperable or significantly inoperable the normal metal dependent genetic circuitry that occurs *in vivo* and causes in whole or in part the prokaryote carrying such a recombinant genetic complement to become less infectious or non-pathogenic. This property renders the recombinant prokaryote suitable for use as an immunogen to be formulated into a vaccine.

10 BEST MODE OF CARRYING OUT THE INVENTION

To prepare the compositions of the present invention, a determination is made as to whether or not the species of interest regulates virulence determinant expression as a function of available metal ion concentrations. This can be done, for example, by screening protein from the bacteria of interest with an antibody for DtxR or other DtxR like proteins to ascertain if a homologous repressor exists in the species. This can also be accomplished using specialized techniques like gel mobility shift assays or the method disclosed in Sun et al, PNAS 95:14985-14990 (1998), or more common gene expression monitoring methods such northern analysis, and PCR. If the species of interest employs a DtxR type repressor than the expression of this repressor can be elucidated by one of the aforementioned methods and the techniques described here can be employed to build a recombinant attenuated strain for vaccine purposes.

Preferred prokaryotes are Gram positive bacterial species, and particularly those listed below. These species contain DtxR like metal dependent repressors. Specific examples include:

S. pneumoniae	S. agalactia	S. equisimilis
S. meningitis	S. bovis	S. anginosus
S. pyogenes	S. salivarius	S. sanguis
S. suis	S. mutans	Enterococcus faecalis
Staphylococcus species		
S. aureus	S. epidermitis	

Mycobacteria species

M. tuberculosis	M. avium complex	M. kansasii
M. leprae	M. scrofulaceum	M. fortuitum
M. ulcerans	M. marinum	M. bovis
M. microtii	M. africanum	M. paratuberculosis

Actinomyces species

A. pyogenes	A. israelii	A. bovis
A. viscosus	A. hordeovulneris	A. gerencseriae
A. naeslundii	A. odontolyticus	and others

Listeria monocytogenes**Propionibacterium acnes****Erysipelothrix rhusiopathiea**

The repressor which typifies the class of genetic regulators in the above listed bacteria is the diphtheria toxin repressor DtxR in *C. diphtheriae*, the causative agent of diphtheria. DtxR is a metal dependent repressor which under limiting concentrations of metal ions becomes inactivated permitting the derepression of a number of virulence genes including diphtheria toxin. This pattern of gene expression is common to both Gram positive and Gram negative bacteria. In Gram positive bacteria, DtxR or DtxR homologues appear to be important metal dependent regulators whereas in Gram negative bacteria, Fur is the significant metal dependent regulator. Some species of pathogen appear to contain both DtxR and Fur like metal dependent repressors. In each case, the presence of repressor bound metal ion is critical for appropriate activity of the repressor which coordinates the repression of gene expression *in vivo*.

The prokaryotes of the present invention having a dominant, metal ion independent or partially metal ion independent mutant of the repressor (such as the diphtheria toxin repressor gene *DtxR* or a *DtxR*-homolog e.g., IdeR, SirR) will render the pathogen unable to effectively establish a full infection. (Hill et al, (1998) Infection and Immunity 66: 4123-4129; and Dussurget et al (1996) Molecular Microbiology 22 536-544; and Pohl et al (1999) J. Molecular Biology 285 1145-1156). Apart from the targeting vector sequences or plasmid DNA used to generate

the attenuated strains and the mutant repressor gene, the recombinant prokaryote is in all other aspects identical genetically to the wild-type organism. The presence of the dominant iron independent repressor results in a phenotypic change in the organisms virulence.

5 In preferred embodiments, the exogenous DNA comprises a sequence encoding a dominant, metal-independent DtxR or a functional fragment, variant or homologue thereof (collectively referred to as "a DtxR protein"). DtxR is a metal iron-dependent DNA-binding protein having a deduced molecular weight of 25,316 and which functions as a global regulatory element for a variety of genes on the *C.*
10 *diphtheriae* chromosome. See Tao *et al.*, Proc. Natl. Acad. Sci. USA 89:5897-5901 (1992); Schmitt *et al.*, Infect. Immun. 59:1899-1904 (1994). For example, *DtxR* regulates the expression of the diphtheria toxin structural gene (*tox*) in a family of closely related Corynebacteriophages. The repressor has also been shown to regulate a number of other iron dependent genes. The gene for DtxR and a number of DtxR
15 homologues have been cloned and sequenced. A number of detailed structural and functional studies have been performed to analyse DtxR. See Boyd *et al.*, Proc. Natl. Acad. Sci. USA 87:5968-5972 (1990); Schmitt *et al.*, *supra*. *DtxR* is activated by divalent transition metal ions (*e.g.*, iron). Once activated, it specifically binds the diphtheria *tox* operator and other related palindromic DNA targets. See Ding *et al.*,
20 Nature Struct. Biol. 3(4):382-387 (1996); Schiering *et al.* Proc. Natl. Acad. Sci. USA 92:9843-9850 (1995); White *et al.*, Nature 394:502-506 (1998). DNA sequences encoding DtxR from various *C. diphtheria* strains are defined by accession numbers M80336, M80337, M80338, and M34239.

Functional fragments or variants of *DtxR*, when activated, retain their
25 binding activity to the *tox* operator (or a functional fragment thereof) and/or the *DtxR* consensus binding sequence. *DtxR* fragments and variants can be identified by standard techniques such as mutagenesis. Tao *et al.*, Proc. Natl. Acad. Sci. USA 90:8524-8528 (1993) identified important residues for DtxR function and analysis. Other variants are disclosed in Tao *et al.*, Mol. Microb. 14(2):191-197 (1994). Tao
30 discloses that some *DtxR* alleles have different amino acid sequences, *e.g.*, the *DtxR* allele from strain 1030(-) of *C. diphtheriae* was found to carry six amino acid

substitutions in the C-terminal region, none of which affected the iron-dependent regulatory activity of *DtxR* (1030) (Tao II). See also Boyd *et al.*, J. Bacteriol. 174:1268-1272 (1992) and Schmitt *et al.*, Infect. Immun. 59:3903-3908 (1991). Thus, *DtxR* fragments and variants may be mutagenized to an iron-independent genotype.

Many other bacterial species employ regulatory circuits and repressor proteins that exhibit high degrees of sequence similarity to *DtxR*. (Posey *et al* (1999) Proc Natl Acad Sci 96 10887-10892, Que and Helmann (2000) Molecular Microbiology 35 1454-1468, Kitten *et al.*, (2000) Infect and Immun 68 4441-4451, Manabe (1999) Proc Natl Acad Sci 96 12844-12848. Thus, dominant, metal-independent *DtxR* homologues may also be employed in the methods of the present invention. Iron dependent regulator (*IdeR*), isolated from *Mycobacterium tuberculosis*, has been found to share 60% amino acid homology with *DtxR*. See Schmitt *et al.*, Infect Immun. 63(11):4284-4289 (1995). See also Doukhan *et al.*, Gene 165(1):67-70 (1995), which reports and references *DtxR* homologs in *Mycobacterium smegmatis* and *Mycobacterium leprae*. *DtxR* homologues have been cloned in other gram-positive organisms including *Brevibacterium lactofermentum* and *Streptomyces lividans*. See Oguiza *et al.*, J. Bacteriol. 177(2):465-467 (1995); Günter *et al.*, J. Bacteriol. 175:3295-3302 (1993); and Schmitt *et al.*, Infect. Immun. 63:4284-4289 (1995). Staphylococcal iron regulated repressor (*SirR*), native to *Staphylococcus epidermitis*, is another known *DtxR* homologue. These proteins bear a common feature they share a remarkably high sequence similarity in the respective N-terminal 139 amino acid regions, especially those amino acids involved in DNA recognition and transition metal ion coordination.

A collection of accession numbers for sequences that are either homologous to *DtxR* or contain a consensus tox O/P is presented in Table 1. See <http://www.ncbi.nlm.nih.gov/BLAST> and <http://www.ncbi.nlm.nih.gov/unfinishedgenomes.html>. See also, Altschul, *et al.*, J. Mol. Biol. 215:403-410 (1990); Gish, *et al.*, Nature Genet. 3:266-272 (1993); Madden, *et al.*, Meth. Enzymol. 266:131-141 (1996); Altschul, *et al.*, Nucleic Acids Res. 25:3389-3402 (1997); and Zhang, *et al.*, Genome Res. 7:649-656 (1997). This

- high degree of sequence similarity and homology is indicative of a widely conserved metal ion dependent regulatory pathway employing *DtxR*-family repressors. It is noteworthy that many important human and animal pathogens are present in this collection of bacteria. Dominant metal independent repressors can be generated and
- 5 introduced into a prokaryote. Such mutants alter virulent phenotype *in vivo* and may be used as vaccines.

Table 1

DtxR Homologs and Species with DtxR Binding Sites

	Pathogenic Human/Veterinary Applications		Other	
5	CAA67572	S. epidermitis	L35906	C. glutamicum
	Gi 1777937	T. pallidum	Z50048	S. pilosus
	CAA15583	M. tuberculosis	Z50049	S. lividans
	U14191	M. tuberculosis		
	L78826	M. leprae		
10	M80336	C. diphtheriae		
	M80337	C. diphtheriae		
	M34239	C. diphtheriae		
	M80338	C. diphtheriae		
	AAD18491	C. pneumoniae		
15	Gi 3328463	C. trachomatis		
	TIGR 1280	S. aureus		
	OUACGT	S. pyogenes		
	Sanger 518	B. bronchoseptica		
	Sanger 1765	M. bovis		
20	Sanger 520	B. pertusis		
	WUGSC	K. pneumoniae		
	TIGR 1351	E. faecalis		
	AE000783	B. burgdorferi		
	TIGR1313	S. pneumoniae		
25	Snager 632	Y. pestis		
	AE001439	H. pylori		
	TIGR 1752	V. cholera		
	TIGR1097	C. tepidum		
30	U14190	M. smegmatis		
	Gi 2621260	M. thermoautotrophicum		
	Gi 2622034	M. thermoautotrophicum		
	M50379	M. jannaschi		
	Q57988	M. jannaschi		
35	O33812	S. xylosum		
	Gi 264870	A. fulgidus		
	Gi 2648555	A. fulgidu		
	Gi 2650396	A. fulgidus		
	Gi2650706	A. fulgidus		
40	BAA79503	A. permix		
	CAB49983.1	P. abyssi		
	BAA30263	P. horikoshi		
	AL109974	S. coelicolor		
45	L35906	B. lactofermentumStanford 382	S. meliloti	
	TIGR 76	C. crescentus		
	TIGR 24	S. putrificacicus		
50	AE000657	A. aeolius		
	TIGR 920	T. ferrooxidans		

Preferred gram positive pathogenic bacteria include *Mycobacterium bovis*, *Mycobacterium leprae*, *Mycobacterium paratuberculosis*, *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Staphylococcus aureus*, *Staphylococcus epidermitis*, *Streptococcus mutans* and *Streptococcus pneumoniae*. The preferred
5 dominant DtxR (or homologue) repressors are metal independent and contain a single amino acid change to convert the native, metal-dependent repressor to metal independent regulation.

A preferred metal independent repressor is the mutant E175K DtxR. This same substitution can be made in DtxR fragments and variants. In addition,
10 DtxR homologues that exist in other bacterial species may be mutated at the corresponding position. Figure 1 displays the degree of amino acid homology between DtxR and to homologous proteins, IdeR from *Mycobacterium tuberculosis* and SirR from *Staphylococcus epidermitis*. As shown in fig. 1, the single amino acid at residue E175K in DtxR, the glutamic acid is conserved in IdeR hence a 177K
15 mutation of IdeR would most likely have the analogous functional implications. Dussurget et al (1996) Molecular Microbiology 22 536-544 and Pohl et al(1999) J. Molecular Biology 285 1145-1156).

Figure 2 presents a comparison of IdeR/SirR homologues found in other mycobacterium that cause significant disease including; *M. tuberculosis*, *M smegmatis*, *M. leprae* and a SirR clones from *M. tuberculosis*. Shown in bold is the
20 conserved glutamic acid in the C-terminal region of these repressors that can potentially be mutated to yield an iron independent version of each of these repressors. Thus, corresponding mutations would be expected to result in a metal-independent genotype. Figure 3 illustrates the amino acid sequence homology of a
25 number of homologous DtxR type repressors. These repressors differ slightly in their sequence length and the ClustalW program used to carry out a logical alignment adjusts for these differences. See NCBI [www.ncbi.nlm.nih.gov] web site and Baylor College of Medicine Search Launcher[<http://gc.bcm.tmc.edu>] for details. Indicated by Bold 'E' is the highly conserved glutamic acid residue in the C-terminus
30 of these repressors which is a possible target for generating metal ion independent versions of each of these repressors.

A combination of standard techniques may be used to make other dominant, metal-independent DtxR proteins, namely mutagenesis followed by tests to determine if a given mutant binds the corresponding operator. A number of theoretical mutations can be incorporated which will convert the repressor to be metal-independent. To generate repressor DNA clones with a random distribution of mutated bases, any of several saturation mutagenesis techniques can be utilized. DNA, plasmid preparation, and DNA sequence analysis are performed according to standard methods. The saturation of random mutations throughout the length of repressors DNA of interest introduces random changes in amino acid sequence throughout the encoded protein that potentially can confer a metal independent phenotype on the mutant repressor.

Several approaches can be pursued to generate effective vaccines relying upon DtxR. In preferred embodiments, DtxR homologues are identified in a species of interest. This can be achieved by performing PCR on genomic DNA from the species of interest using primer sets compatible to conserved domains within the DtxR family. Specific sequences may be composed of degenerate primers flanking the iron binding domain, or the helix-turn-helix domain. Cloning may also be performed from phage libraries using DtxR conserved regions as probes, utilizing the PSTD system or by *in silico* searches and de novo *in vitro* synthesis.

The DtxR has a domain structure which is composed of a helix-turn-helix domain, and N-terminal iron binding domain. There are a number of additional highly conserved sequences including a proline rich region which lies between the HLH and C-terminal domain. The polymerase chain reaction allows the amplification of any DNA target from a population of DNA molecules if compatible oligonucleotide primers can be identified. While a number of software programs are available to assist in the design of effective primers, optimal primer sets often require empirical determination.

Cloning of DtxR homologues from any given species can be achieved by mixing primers with genomic DNA isolated from the species of interest in the appropriate ratios in the presence of free oligonucleotides, optimized buffer conditions and the TAQ polymerase (or a suitable version of a thermostable

polymerase) and cycling the reaction conditions with the aid of an automated thermocycler. The PCR reaction generates a series of products that represent target DNA sequences that are bounded by sequences homologous to the selected 5' and 3' primers. Thus, the selection of primers which lie within conserved regions of DtxR will likely bind under the appropriate conditions to homologous DNA that will likely represent a gene or domain similar to that of DtxR.

DtxR homologues may further be identified by screening genetic libraries of a given species created in *E. coli*. Pathogen libraries can then be screened by radio-labeled probes generated from DtxR clones, oligonucleotides from the DtxR sequence, or protein assays using antibodies directed against DtxR.

Sun, *et al.* have developed a screen can be used as a positive selection assay for DtxR homologues. See Sun, *et al.*, PNAS 95:14985-14590 (1998). This screen requires only the generation of a gDNA library from the species of interest be transformed into the appropriate background host. Colonies which appear upon selection by chloramphenicol can only arise if a functional DtxR like protein is being expressed from the cloned gDNA fragment. Once a clone has been identified, sequencing and sequence analysis will reveal a gene which has at least partial sequence homology to DtxR and functional equivalence based on the PSDT screen developed by Sun *et al.*

Once identified, these clones become the substrates from which a vaccine strain is assembled. Two approaches are preferred. The first approach is a knock-in approach which is coupled with *in vitro* mutagenesis or PCR mutagenesis. The knock-in approach is focused on generating strains having dominant activated repressors. These strains contain a defective or altered copy of DtxR or a DtxR homologue that containing at least one but up to several mutations resulting in a repressor that recognizes and binds the toxAPO or suitable cognate binding sites in both the presence and absence of iron (or the appropriate metal ion). These metal ion independent mutants are cloned into suitable vectors (e.g., targeting vectors having a selectable marker and restriction sites) to develop strains expressing dominant activated DtxR constitutively. These plasmids are used to generate knock-in vaccine strains, essentially altered forms of the wild-type virulent strain differing in only the

presence of a dominant activated DtxR or DtxR-homologous repressor. The constructs can also be used in gene replacement strategies in which the mutant metal independent repressor gene replaces the endogenous wild type gene. (Howard et al (1995) Gene 166 181-182, Jacobs et al (1991) Mthds in Enz 204 537-555., Rubin et al (1999) Proc Natl Acad Sci 1644-1650), Caparon and Scott (1991) Mthds in Enz 204 556-586, Norgren, Caparon and Scott, (1989) Infect and Immun 57 3846-3850, Biswas et al, (1993) J Bact. 175 3625-3635. Such a strain is effectively avirulent since its ability to up-regulate iron dependent virulence genes has been crippled.

Metal ion independent clones can be sequenced to identify the specific amino acid changes that confer the iron independent phenotype. Again, a preferred mutant is characterized by a change from glutamic acid to lysine at position 175 in native DtxR. In general, however, multiple mutations are also embraced by the present invention. Multiple mutations are advantageous because they greatly decrease the likelihood of reversion to wild-type function. This likelihood becomes statistically more akin to that of a strain which has all of its iron regulated genes knocked out. Multiple mutations either clustered or distributed through out the repressor can result in the same phenotype. For example, a double mutant DtxR with the replacement of asparagine at position 130 with glycine and the glutamine at position 181 with arginine was also identified with a metal independent phenotype. In addition a mutant with an intermediate phenotype was also identified having a total of six mutations[valine 5 to isoleucine, aspartic acid 110 to glutamic acid, valine 112 to phenylalanine, isoleucine 153 to threonine, aspartic acid 197 to glutamic acid, threonine 220 to alanine]. These mutations produced a partially metal dependent phenotype. Thus, the present invention also embraces the use of partially metal ion-independent repressors. It is believed that expression of these repressors allow the partial colonization of the host which promotes the development of a robust protective immune response. The metal dependent phenotype can be readily revealed by linking a readily assayed reporter gene to the *tox O/P* in *E. coli* and a assessing reporter function under metal and metal conditions as described by Sun, *et al*.

More generally, however, the DtxR mutants of the present invention are not limited to those characterized by single or multiple amino acid substitutions.

Insertion and deletion mutants are also contemplated. These mutants may be identified using the instantly disclosed techniques as well.

Strains and putative mutants can be tested for metal independent phenotype by several approaches including the PSTD screen described in Sun *et al.*

- 5 Additional methods of determining if conversion to iron independent phenotype include computer modeling, structural analysis, dimerization analysis by gel electrophoresis, DNA binding by electrophoretic mobility shift assay, transcriptional profiling, tissue culture and in vivo virulence assays. Ultimately, *in vivo* screening will be required to determine if the iron independent phenotype is stable in a host
- 10 environment and to determine if virulence is attenuated. Attenuated virulence can be defined by biochemical, physiological and immunological markers but will minimally include an assessment of ED₅₀ or LD₅₀ in wild-type, knock-in, replacement and recombinant strains.

- A second rational mutagenesis strategy can be utilized to generate
- 15 metal-independent mutants. This strategy relies upon the conserved domain structure of the DtxR family of repressors. Several DtxR iron independent mutants have been identified and published. These mutants define two classes of iron independent mutations which are likely to alter iron dependent regulation in DtxR homologues. Site directed mutagenesis of the analogous amino acid residues in DtxR homologues
- 20 may have the same iron independent phenotype. In addition as the data from structural studies grows it will likely be possible to construct mutants that replace amino acids involved in the coordination of iron binding or dimerization that result in an iron independent phenotype.

- Mutagenesis is then carried out to generate a library of the desired
- 25 mutants of this DtxR homologue of interest. Identification of iron independent mutants from this population is achieved by using the PSTD system. Using the cloned and mutant homologue genes, one can select for the growth of colonies in the PSTD strains in the presence of dipyriddy (DP). Dipyriddy chelates iron from the media and therefore leads to the disassociation of DtxR homologues from the
- 30 regulatory regions of the genetic elements in the PSTD screen. As a result, all iron

dependent repressors will not be able to survive chloramphenicol selection, in contrast iron independent mutants will grow.

5 Mutants of DtxR are generated in accordance with standard techniques. Polymerase chain reaction (PCR) mutagenesis of the *dtxR* gene is described in Vartanian *et al.* [Vartanian, J.-P., Henry, S., & Wain-Hobson, S. (1996) Hypermutagenic PCR involving all four transitions and a sizeable proportion of transversions. *Nucleic Acid Res.*, 24, 2627-2631.]. Briefly, *Bgl*II-tagged primers 1515 (5'-ACCAGATCTGCCGAAAACTTCGA-3') and 1516 (5'-ACCAGATCTCCGCCTTTAGTATTTA-3') were used to PCR amplify *dtxR* from 10 plasmid pRDA which carries the wild type *dtxR* operon. The products of the amplification were then digested with *Bgl*II and ligated either into *Bgl*II-linearized pSC6M1 and transformed into *E. coli* TOP10/ λ RS65T, or ligated into *Bam*HI digested pBR322 and transformed into *E. coli* TOP10/ λ RS65T/pSC6. Iron-independent mutants of DtxR were then selected on LB agar plates supplemented 15 with Cm and DP in accordance with the procedure described in Sun, *et al.*

Broadly speaking, these mutations should conserve the structural integrity and maintain the ability of the repressor to bind and repress gene expression through the consensus or near consensus *tox* P/O sites. Bacterial clones containing mutagenized *dtxR* can be analysed by DNA sequencing and used in functional 20 biochemical assays such as electrophoretic mobility shift assay, native gel analysis and glutaraldehyde crosslinking studies to reveal the activated state of the repressor in question under metal limiting conditions. This can be determined through gel shift analysis or by functional assays, but it is preferably made using the one-step method described by Frigg, *et al* [Sun, L., vanderSpek, J. & Murphy, J. R. (1998)*Proc.* 25 *Natl. Acad. Sci. USA*, 95, 14985-14990]. For gel shift analysis the native *tox* operator (i.e., 5'-ATAATTAGGATAGCTTTACCTAATTAT-3') is a 27 base pair interrupted palindromic sequence upstream of the diphtheria *tox* structural gene can be used as a probe. This sequence features a 9-base pair inverted repeat sequence that is separated by 9 base pairs. See Kaczorek *et al.*, *Science* 221:855-858 (1983); 30 Greenfield *et al.*, *Proc. Natl. Acad. Sci. USA* 80:6853-6857 (1983); Ratti *et al.*, *Nucleic Acids Res.* 11:6589-6595 (1983); and Fourel *et al.*, *Infect. Immunol.*

57:3221-3225 (1989). It overlaps both the -10 region of the *tox* promoter and the transcriptional start sites at -45, -40 and -39 upstream of the diphtheria toxin structural gene. See Boyd *et al.*, J. Bacteriol. 170:5940-5952 (1988). The minimal essential DNA target site, i.e., 5'-GTAGGTTAGGCTAACCTAT-3', is a 19 base pair sequence that forms a perfect palindrome around a central C or G -- is described in Tao and Murphy, Proc. Natl. Acad. Sci. USA 91:9646-9650 (1994). Additional probes are variants of ToxO based on the *DtxR* consensus-binding sequence (5'-ANANTTAGGNTAGNCTANNCTNNNN-3'). Variants are defined by the following sequence: 5'-TWAGGTTAGSCTAACCTWA-3'. Thus the function of the repressor and mutant can be defined by recognition and binding or regulation of gene expression via the sequences or variants described above.

Once a dominant, metal-independent *DtxR* DNA clone is identified, it can be produced and manipulated in accordance with techniques known in the art. For example, they may be generated using standard chemical synthesis techniques. See, *e.g.*, Merrifield, Science 233:341-347 (1986) and Atherton *et al.*, Solid Phase Synthesis, A Practical Approach, IRL Press, Oxford (1989). Preferably, they are obtained by recombinant techniques. Standard recombinant procedures are described in Sambrook *et al.*, 1989, Molecular Cloning, A Laboratory Manual, Second ed., Cold Spring Harbor, New York, and Ausubel *et al.*, (eds.) Current Protocols in Molecular Biology, Green/Wiley, New York (1987 and periodic supplements). The appropriate sequences can be obtained from either genomic or cDNA libraries using standard techniques. DNA constructs encoding the DNA gene segments may also be prepared synthetically by established methods, *e.g.*, in an automatic DNA synthesizer, and then purified, annealed, ligated and cloned into suitable vectors. Atherton *et al.*, *supra*. Polymerase chain reaction (PCR) techniques can also be used. See *e.g.*, PCR Protocols: A Guide to Methods and Applications, 1990, Innis *et al.* (ed.), Academic Press, New York.

The DNA encoding the mutant metal-independent repressor is operably linked to a promoter element. Preferred promoters include the endogenous *DtxR* promoter or the promoter of the *DtxR* homologue in question or any suitable constitutive promoter functional in the species of interest. the constructs may further

contain an associated selectable marker gene to follow maintenance of the mutant construct. For example in Mycobacterial species a hygromycin resistance gene in an *E. coli* shuttle vector provides great utility for cloning and expression of a mutant IdeR or another dominant metal ion independent repressor [DtxR E175K]. [Bishai *et al* Gene 1995 166:181-182] In other embodiments, the vectors also contain a copy of a gene lethal to the bacterium under the control of the metal dependent regulator. Examples of such genes include antibiotic genes, restriction enzymes, proteolytic enzymes, lethal phage genes or any gene whose product once expressed would kill the bacterium. The presence of this "suicide cassette" further ensures that vaccines containing the prokaryote in live form do not revert to any significant degree and cause disease *in vivo*.

Suitable cloning and expression vectors are readily available from a number of sources. In preferred embodiments, the construct is introduced into the prokaryote by way of a vector, in which case the construct may be formed prior to or upon introduction of the DNA into the vector. A vector for a given species must contain an origin of replication, a selectable marker and a functional promoter by which the mutant metal ion independent repressor can be expressed in the strain of interest. (Howard *et al* (1995) Gene 166 181-182, Jacobs *et al* (1991) Mthds in Enz 204 537-555., Rubin *et al* (1999) Proc Natl Acad Sci 1644-1650), Caparon and Scott (1991) Mthds in Enz 204 556-586, Norgren, Caparon and Scott, (1989) Infect and Immun 57 3846-3850, Biswas *et al*, (1993) J Bact. 175 3625-3635.

The vectors are introduced into the cells in accordance with standard techniques such as transformation, co-transformation, direct transfection (*e.g.*, mediated by calcium phosphate or DEAE-dextran) biolistics and electroporation. The recombinant cells are then cultured via standard techniques. Conditions may vary depending upon the prokaryote (*e.g.*, bacterial species) being used. In general, culturing is continued from about 24 to 48 hours at a temperature between about 30 and about 39°C, preferably 37°C. The recombinant cells are cultured in an appropriate complete medium containing a selectable marker to assure a pure population. The strains can also be counter engineered as described below so that if significant levels of the metal independent repressor is not produced a suicide gene

will be de-repressed resulting in the death of the vaccine strain *in situ*. Iron is an essential element for both the bacterial pathogen and its animal host; thus, successful competition for this element is an essential component of the infectious process. The concentration of free iron in the mammalian host available to an invading bacterial pathogen is also extremely limited. As a result, the expression of virulence determinants (e.g., colonization factors, siderophores, hemolysins and toxins) by bacterial pathogens is regulated by iron. Accordingly, the vaccine cultures must be propagated in a complete medium containing iron and other divalent metal cations to facilitate their proliferation.

The vaccines of the present invention may be used in a wide variety of vertebrates, particularly man and domestic animals such as bovine, ovine, porcine, equine, caprine, domestic fowl, *Leporidae*, or other animals that may be held in captivity or may be a vector for a disease affecting a domestic vertebrate.

Pathogens of interest include any specie of microorganism which causes disease and relies entirely or partially upon a repressor mediated regulation of metal-dependent virulence. The present invention relates to methods of vaccinating a host with live recombinant bacteria to elicit protective immunity in the host. The recombinant vaccine can be used to produce humoral antibody immunity, cellular immunity (including helper and cytotoxic immunity) and/or mucosal or secretory immunity.

The manner of application of a vaccine strain may be varied widely, any of the conventional methods for administering an attenuated vaccine being applicable. These include aerosol applications, oral applications, in drinking water, on a solid physiologically acceptable base, or in a physiologically acceptable dispersion, parenterally (e.g., subcutaneously, intramuscularly, intravascularly or intraperitoneally), by injection, by *in ovo* inoculation or the like. The dosage of the vaccine (e.g., number of prokaryotic cells, number of administrations, period of administration, etc.) will vary according to the vaccine strain used and the species, age, and size of host to be protected. Persons skilled in the art will be able to determine the dosage to be administered so as to provide a sufficient immune

response. The recombinant prokaryotes in the composition may be "live" or in "killed" form, as these terms are commonly used in the vaccine art.

The formulation of vaccine strain compositions may also vary widely. Pharmaceutically acceptable vehicles such as water are expected to be useful for oral
5 administration. Other such vehicles including normal saline may be used for parenteral, cloacal or other routes of administration. The vaccine compositions may also be admixed with food for some applications.

The following example is not intended to limit the scope of the invention in any manner.

10

Example 1

Attenuation of Virulence in *Mycobacterium tuberculosis* Expressing a Constitutively Active Iron Repressor

This example describes the construction of a candidate strain which is severely attenuated with respect to the parent wild type strain yet which persists long
15 enough to allow the host to mount an immune response.

Summary

With over one-third of the world's population latently infected with *Mycobacterium tuberculosis*, the global burden of tuberculosis is staggering. The emergence of multi-drug resistant strains and the increased susceptibility of the HIV-
20 infected further highlights the need for elucidation of the molecular pathogenesis of *M.tuberculosis* and its virulence genes.

Iron plays a critical role in the regulation of virulence of many bacterial pathogens. (1) In tuberculosis, there is indirect clinical and *in vitro* evidence that iron regulation is important to the virulence of this microbial pathogen. (2-5)
25 Iron is an essential nutrient for the survival of most organisms and has played a central role in the virulence of many infectious disease pathogens. Mycobacterial IdeR is an iron-dependent repressor that shows 80% identity in the functional domains with its corynebacterial homologue, DtxR. In a novel approach to attenuation, *Mycobacterium tuberculosis* has been transformed with a vector
30 expressing an iron-independent, positive dominant, corynebacterial *dtxR* hyperrepressor, DtxR(E175K). Western blots of whole cell lysates of *M.*

tuberculosis expressing the *dtxR*(E175K) gene revealed the stable expression of the mutant protein in mycobacteria. BALB/c mice were infected by tail vein injection with 2×10^5 organisms of wild type or *M.tuberculosis* transformed with the *dtxR* mutant. At 16 weeks, there was a 1.2 log reduction in bacterial survivors in both spleen ($p=0.0002$) and lungs ($p=0.006$) with *M.tuberculosis* DtxR(E175K). A phenotypic difference in colonial morphology between the two strains was also noted. A computerized search of the *M.tuberculosis* genome for the palindromic consensus sequence to which DtxR and IdeR bind, revealed six putative "iron boxes" within 200 base pairs of an open reading frame. Using a gel shift assay, it was shown that purified DtxR binds to the operator region of five of these. Attenuation of *Mycobacterium tuberculosis* can be achieved by the insertion of a plasmid containing a constitutively active, iron-insensitive repressor, DtxR(E175K), which is a homologue of IdeR. The results demonstrate that IdeR controls genes essential for virulence in *M.tuberculosis*.

In a phylogenetically related organism, *Corynebacterium diphtheriae*, iron depletion results in the derepression of virulence genes such as the diphtheria toxin (*tox*) gene by DtxR (diphtheria toxin repressor). The corynebacterial DtxR has a homologue in *M.tuberculosis*, IdeR (iron-dependent repressor). In the amino terminal 140 amino acids that contain the Fe^{2+} and DNA-binding domains of DtxR, IdeR shares 80% identity with DtxR. (6) In 1995, *ideR* was first described by Doukhan *et al.* in conjunction with the *sigA sigB* cluster of genes. (7) Subsequently, the ability of mycobacterial IdeR to bind to the corynebacterial *tox* operator region in a metal ion-dependent manner was demonstrated by gel shift assay. (8) Mutation of *ideR* in *M.smegmatis* resulted in derepressed siderophore production in high iron conditions. (9) These findings parallel those described in corynebacterial *dtxR* and suggest that the homology between these two genes may allow for cross-genus functional complementation.

Using a positive genetic selection system to clone *dtxR* alleles, Sun *et al.* isolated and characterized a series of DtxR mutants created by PCR mutagenesis. (10) One of the mutants which bound to the *tox* operator (*toxO*) and constitutively repressed reporter gene expression in an iron-independent manner was characterized

and found to have a single amino acid substitution of lysine for glutamic acid at position 175 (DtxR(E175K)). In merodiploid strains harboring both wild type *dtxR* and mutant *dtxR*(E175K) genes, Sun *et al.* found the mutant to be dominant over the wild type allele.

5 *Methods*

Strains, Plasmids, and Cultures The bacterial strains and plasmids used in this study are listed in Table 2. (10-14) *Escherichia coli* cultures were grown in Luria broth or Luria agar supplemented with ampicillin (100g/ml) or hygromycin (200g/ml). *M.tuberculosis* CDC 1551 and *M.smegmatis* cultures were grown in
10 standard Middlebrook 7H9 broth (Difco), supplemented with albumin dextrose complex (ADC), 0.1% glycerol, and 0.05% Tween 80 at 37°C in roller bottles. (15)

Construction of *dtxR* (E175K) Shuttle Vector Plasmid A 1.5 kb *Bam*HI-*Hind*III fragment of DNA from pSDM2 was cloned into pNBV1. The resulting recombinant plasmid, pNBV1/SAD was cloned in *E.coli* DH5 and purified
15 using the Qiagen system (Qiagen, Chatsworth, CA). (16) Purified plasmids were then electroporated into *M.tuberculosis* CDC1551 by standard protocols. (15)

Western Blot Analysis Recombinant *E.coli* and mycobacteria were lysed in 3M urea, 0.5% Triton X-100, 3.25M DTT, 2% Pharmalyte (Pharmacia Biotech, Piscataway, NJ), PMSF (100g/ml) and leupeptin (2g/ml). Using 0.1 mm
20 glass beads, the samples were homogenized twice in a Mini-bead-beater (Biospec Products, Bartlesville, OK) at maximum speed for 1 minute. Samples were centrifuged to remove cellular debris and unlysed cells. After separation by 12% SDS polyacrylamide gel electrophoresis, proteins were transferred to nitrocellulose membranes (Hybond, Amersham, Buckinghamshire, UK) by semi-dry technique
25 (Transblot SD, Hercules, CA) and blocked with 5% non-fat milk in PBS with 0.1% Tween 20 (PBS-T) for 1 hour. Membranes were then incubated overnight in PBS-T with rabbit anti-DtxR polyclonal antibodies at the appropriate concentration at 4°C. (17) After washing, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit antibody diluted in PBS-T for 2 hours. The Supersignal
30 Chemiluminescent Substrate (Pierce, Rockford, IL) was used for autoradiograph development.

Murine Tuberculosis Infection Model 6-8 week-old BALB/c mice

were infected by tail vein injection with 2×10^5 organisms of wild type or *M.tuberculosis* DtxR(E175K). Bacterial infection was monitored over a 119-day period. Colony forming units (CFU) in spleen and lungs were assessed at 4 week intervals by serial dilutions of organ homogenates plated on 7H10 Middlebrook agar containing cycloheximide (50 g/ml), carbenicillin (50 g/ml), trimethoprim (20 g/ml), and polymyxin (200 units/ml). (18)

DNA Gel Shift Binding Assay The DNA migration retardation assay

was performed as previously described. (19) Purified DtxR protein was isolated by methods as described. (20) Radiolabeled DNA iron box fragments were generated by PCR using 100 ng of ^{32}P -end-labeled primer mixed with 150 ng of unlabeled primer and template DNA from gel-purified 100 bp cold fragments containing the iron box of interest. Binding reactions were carried out in 10mM Tris-OAc (pH7.4), 1mM EDTA, 50mM KCl, 1mM DTT, 5% glycerol, 50g/ml calf thymus DNA. Binding reactions were equilibrated for 30 minutes and then loaded onto a non-denaturing 6% acrylamide gel. (21)

Results**Expression of the corynebacterial *dtxR* gene in mycobacteria**

The 1.5 kb corynebacterial DNA fragment cloned in pNBV1/SAD contained 500 bp of 5' non-coding sequences as well as the entire *dtxR*(E175K) open-reading frame. To determine if the corynebacterial mutant *dtxR*(E175K) gene was expressed in mycobacteria, we transformed *M.smegmatis*, a fast growing strain of mycobacteria, with pNBV1/SAD. Whole cell lysates prepared from *M.smegmatis* cultures were separated by 12% SDS PAGE. Figure 4 shows a Western blot developed with polyclonal anti-DtxR antibodies. As illustrated, these antibodies recognize both DtxR and IdeR because of their significant antigenic similarity. Although the deduced molecular mass of IdeR (25.2kDa) differs by only 0.1kDa from DtxR (25.3kDa) we have repeatedly observed anomalous accelerated migration of IdeR in our SDS-PAGE gels in which it runs at 23kDa in spite of its mass of 25kDa. This phenomenon has also been noted by Schmitt *et al.* (8) In preparations from *M.smegmatis* harboring pNBV1/SAD (Fig4, lane 4), two distinct bands appear.

Because *dtxR*(E175K) is expressed from a multicopy plasmid, significantly more DtxR(E175K) protein is made than the chromosomally expressed IdeR. Similar results in *M.tuberculosis* transformed with pNBV1/SAD were also found (results not shown). The *in vitro* growth rate of wild type *M. tuberculosis* was indistinguishable from that of *M. tuberculosis* DtxR(E175K) by the BACTEC radiometric growth monitoring system.

Attenuation of Virulence in *M.tuberculosis* Expressing the Constitutively Active DtxR Hyperrepressor After confirming that the corynebacterial mutant *dtxR* was expressed in transformed mycobacteria, we turned to an *in vivo* animal model to test the effect of the hyperrepressor on virulence. Forty-eight BALB/c mice were inoculated with 2×10^5 CFU of CDC1551 *M.tuberculosis* or *M.tuberculosis* DtxR(E175K) by tail-vein injection. Both animal weights and the tissue burden of surviving bacteria were monitored over time. Mice infected with wild type *M.tuberculosis* began to lose weight beginning at 13 weeks while the *M.tuberculosis* DtxR(E175K)-infected animals initially gained weight, then maintained stable weights for the duration of the experiment. At 17 weeks, there was a statistically significant difference of 1.7 gms ($p=0.006$ by two-tailed t-test) between the wild type and DtxR(E175K) groups.

Figures 5A and 5B show the survival of the two *M.tuberculosis* strains in lungs and spleens of mice over time. At 17 weeks, there was a 1.2 log attenuation in virulence of the DtxR(E175K) expressing strain compared with wild type which was statistically significant in both spleen ($p=0.0002$) and lungs ($p=0.006$). Analysis of the colonies from the mouse tissues at 12 weeks showed that 99% of the colonies were hygromycin resistant indicating maintenance of the pNBV1/SAD plasmid. Histopathologic inspection of spleen and lungs of wild type and DtxR(E175K) expressing strains corroborated our CFU data with fewer visible acid fast bacilli at 17 weeks in histologic sections of mouse organs from animals infected with the *M.tuberculosis* DtxR(E175K) than with the wild type.

Differences in Colonial Morphology Between Strains Colonies of *M.tuberculosis* DtxR(E175K) grown from frozen stocks on 7H10 Middlebrook agar showed no difference in growth rate *in vitro* as compared to wild type CDC1551, but

5 were noted to have a distinct colonial morphology (see figures 6A and 6B). The recombinant strain colonies were rougher and drier-appearing and were more raised and wrinkled than wild type colonies. In addition, yellow pigmentation was also noted in the DtxR(E175K) expressor. Both strains exhibited a spreading phenotype and were crenelated at the periphery.

Identification of Iron Boxes An imperfect palindromic consensus sequence of the "iron box" for DtxR/IdeR has been established by *in vivo* and *in vitro* methods. (8, 22, 23) This consensus sequence is listed at the top of Figure 7. To identify genes that may be regulated by IdeR, we searched the *M.tuberculosis* genome for iron boxes that were in untranslated regions within 200 bp of an open reading frame. We chose two half-site sequences with allowance for a variable number of intervening base pairs for our search. In the 4.41MB of the *M.tuberculosis* genome (24), 58 sequences with acceptable homology to the consensus sequence were identified. Six of these were in untranslated regions and had corresponding downstream open reading frames.

A DNA gel binding assay was used to assess the ability of DtxR to bind to these putative iron-regulated operator regions drawn from the *M.tuberculosis* genome. Figure 8 shows the results of gel binding assays using ³²P-end-labeled 100 bp DNA fragments containing five of the putative iron boxes (IB1-5). Binding of DtxR to the *tox* operator could be abolished with the addition of unlabeled *tox* DNA, but not with nonspecific DNA. All five of these putative iron boxes were bound by DtxR to a similar degree as that observed with the *tox* operator. The iron box upstream of the *narG* homologue, IB6, did not bind to DtxR. (data not shown)

Table 3 identifies the open reading frames (ORF) downstream of these six iron boxes. BLAST searches reveal that these genes encode a PhoP homologue (a transmembrane sensor of a two-component sensor-regulator pair), a homologue of the HtrA serine protease, 16S ribosomal RNA, an alcohol dehydrogenase AdhB, and a homologue of the *M.tuberculosis* 19kDa antigen (a protein shown to be involved in the human immune response to tuberculosis). (25) IB6, which was not shifted by DtxR *in vitro*, appears upstream of a nitrate reductase subunit gene, *narG*.

Discussion

The concentration of free ferrous iron (Fe^{2+}) is extremely limited *in vivo*. For this reason, many pathogenic prokaryotes such as *Vibrio cholerae*, *E.coli*, *Neisseria gonorrhoeae*, and *Corynebacterium diphtheriae* co-regulate virulence gene expression with iron sensing and scavenging systems. (26-28) In *C.diphtheriae*, one such mechanism of iron regulation relies on a repressor, DtxR, which binds to a specific palindromic sequence in the operator regions of the genes that it controls. (29) In low iron states, the metal-ion triggered conformational change that allows it to bind to the DNA is disrupted, the repressor loses affinity for the operator site, and gene expression occurs. Recently, a positive dominant DtxR(E175K) mutant unresponsive to iron was generated by random PCR mutagenesis using a genetic selection system. (10)

Significant amino acid identity between corynebacterial DtxR and mycobacterial IdeR has been described. In the amino terminal 140 amino acids there is a DNA binding helix-turn-helix motif, a primary metal ion binding site and a protein-protein interaction domain. Corynebacterial DtxR and mycobacterial IdeR share 80% amino acid identity in this portion of both proteins. Evidence of functional homology between IdeR and DtxR has been shown previously by Schmitt *et al.* (8)

The results show that the positive dominant DtxR(E175K) iron-independent repressor is expressed in the phylogenetically related mycobacteria. Furthermore, we have shown that it is dominant and constitutively attenuates *M.tuberculosis* in a murine model of infection. Rational attenuation of *M.tuberculosis* provides the opportunity to define specific virulence factors of the organism and the development of live vaccines superior to BCG. However, gene replacement has proven difficult in *M.tuberculosis* due to high rates of illegitimate recombination. Addition of a dominant mutant gene is technically simpler than gene replacement in *M.tuberculosis* and permits comparison of a defined merodiploid strain with an isogenic wild type strain. There are reports of *E.coli* genes introduced into other bacteria to regulate the expression of endogenous genes. In a paper by de Henestrosa *et al.*, a mutant *E.coli* *recA* gene produced aberrancies in SOS gene

induction when expressed in heterologous gram-negative systems. (30) Although a *M.tuberculosis* strain containing empty plasmid was not compared with wild type *M.tuberculosis*, the *in vitro* Bactec comparison showed no differences in the rate of growth of the mutant strain as compared to wild type. In addition, studies of deletion mutants have shown that plasmid complementation fully restores virulence suggesting that there is little cost to the organism to maintain the plasmid. (31, 32) This strain is the first example of the use of a dominant positive gene from another pathogenic prokaryote to attenuate the virulence of *M.tuberculosis*.

Animal models have shown that inactivation and clearance of virulent *M.tuberculosis* in liver and spleen is effectively accomplished, but that the same cell-mediated immune mechanisms appear relatively ineffective in lungs. These data point to a difference in the intracellular microenvironment of the lung granuloma. (33) It has been postulated from BCG and H37Ra data that avirulent or attenuated strains lack the genes required for effective growth within lung phagocytes. (34) The data suggest that IdeR may regulate genes important for *M.tuberculosis* survival late in lung infection as attenuation seems to increase dramatically at 12 weeks after infection. This may correlate with the onset of granuloma formation in mouse lungs and the need for *M.tuberculosis* to scavenge iron from extracellular rather than intracellular sources. (35)

The *ideR* gene has been found in *M.tuberculosis*, *M.bovis*, and *M.smegmatis*. In *M.smegmatis*, an *ideR* mutant showed defective regulation of siderophore biosynthesis. (9) Potential IdeR binding sites upstream of exochelin biosynthesis genes such as *fxbA* have recently been confirmed. (36, 37) In addition, several IdeR recognition sequences have been identified using computer searches of the *M.tuberculosis* genome. (38) We have similarly identified 6 potential IdeR-binding sites in *M.tuberculosis*, 5 of which demonstrated significant binding with DtxR in a gel shift assay. We postulate that the sixth sequence was unable to bind in our *in vitro* assay because of incorrect spacing between the two relatively well-conserved half-sites. We used DtxR rather than IdeR in this gel-shift assay because we specifically sought to identify genes responsible for the attenuated phenotype of *M.tuberculosis* DtxR(E175K). The predicted ORF downstream of IB-1 encodes a

homologue of *phoP*, a phosphotransfer response regulator. A number of two-component pairs have been shown to regulate virulence pathways in bacterial pathogens. These include BvgA/BvgS in *Bordetella pertussis*, VanR/VanS in *Enterococcus faecium*, PhoP-PhoQ in *Salmonella typhimurium*, and OmpT/EnvZ in *Shigella flexneri*. (39, 40) In *M.tuberculosis*, a two-component pair, *mtrA-mtrB*, has been previously described and appears to play an intracellular role as expression of *mtrA* increases upon entry into macrophages. (41) Furthermore, *phoP*- mutants in *Salmonella* are unable to synthesize many of the proteins expressed on interaction with macrophages. (42) Downstream of IB-2 is *adhB*, an alcohol dehydrogenase. In *Salmonella typhimurium*, it has been postulated that alcohol dehydrogenase genes such as *eutG* may confer a protective role from reactive aldehyde intermediates associated with inflammatory cell activation. (43)

IB-3 lies upstream of an ORF homologous to a HtrA-like serine protease which, in *E.coli* are thought to be required for growth of the organism at high temperature, and may play a role in degrading abnormal proteins within the periplasm. (44, 45) It is a known virulence factor in several organisms including *Salmonella typhimurium*, *Yersinia enterocolitica*, *Brucella abortus*, and *Brucella melitensis*. (46-49) In an animal protection model, a *Salmonella typhimurium htrA* mutant is attenuated and a safe and immunogenic live vaccine strain in mice. (50) Both *Mycobacterium avium* subsp. *paratuberculosis* and *M.tuberculosis* have putative serine proteases with significant homology to HtrA. (24, 51)

IB-4 lies upstream of *rrnA*, a 16S rRNA gene which has been shown to be part of a group of rDNA operons in both slow and fast-growing mycobacteria with hypervariable multiple promoter regions (HMPR). The *M.tuberculosis rrnA* operon has 2 promoters one of which is conditionally induced suggesting complex regulation of this essential gene. (52)

Our results indicate that a dominant positive corynebacterial *dtxR* allele attenuates the virulence of *M.tuberculosis* in a murine model. These data implicate the *M.tuberculosis* IdeR repressor as a regulator of genes essential for full virulence.

Table 2:

Strain/Plasmid		Genotype/Description	
Source/Reference			
Plasmids			
5	(12)	PNBV1	<i>E.coli</i> -mycobacterial shuttle vector
		Ap ^R , Hy ^R	
10	(10)	pSDM2	pSC101-derivative containing the
		<i>dtxR</i> (E175K) gene, Km ^R	
15		PNBV1/SAD	PNBV1 containing the
	<i>dtxR</i> (E175K)	this paper gene, Ap ^R , Hy ^R	
Strains			
20	(14)	<i>E.coli</i> DH5	F ⁻ <i>recA1</i> , <i>hsdR17</i> , <i>thi-1</i> , <i>gyrA96</i> ,
		<i>supE44</i> , <i>endA1</i> , <i>relA1</i> , <i>recA1</i> , <i>deoR</i> , (<i>lacZYA-argF</i>) U169 (80 <i>lacZ</i> M15)	
25	(13)	<i>M.smegmatis</i> mc ²⁶ 1-2C	transformable variant of mc ²⁶
	(11)	<i>M.tuberculosis</i> CDC1551	virulent recent clinical isolate
30		<i>M.tuberculosis</i> DtxR(E175K)	pNBV1/SAD
		this paper	
Hy hygromycin, Ap ampicillin, Km kanamycin			
Table 3: Iron Boxes (IB)			

Table 3: Iron Boxes (IB)

Name	Downstream ORF	Accession Number	Description
IB-1	PhoP homologue	Rv0761c	Two-component phosphotransferase

			regulatory protein
IB-2	AdhB	Rv0757	Alcohol dehydrogenase
IB-3	HtrA homologue	Rv0983	Serine protease, HtrA-antigen family
IB-4	RrnA	MTB00368	16S ribosomal RNA protein
IB-5	Hypothetical protein	Rv3764c	Predicted ORF with 26% similarity to <i>M.tuberculosis</i> 19kDa antigen beginning at base 4,210,314

Example 2

Attenuation of Staphylococcus Infection in a Murine Model

In Example 1 Applicants demonstrated the ability of *in vitro* constructed and tested metal ion independent mutant gene of a normally metal ion dependent transcriptional repressor to attenuate virulence in a pathogenic bacterium of a different species. The metal ion dependent repressor was from *C. diphtheriae* and the pathogenic species was *M. tuberculosis*. The sequence homology of DtxR and the endogenous mycobacterial DtxR-like repressor IdeR is approximately 60% [approaching 90% in the N-terminal half of the repressors]. In example two, applicants have expanded this observation to demonstrate that a dominant metal ion independent repressor such as the mutant E175K DtxR is capable of attenuating virulence across wider species differences in a bacterial strain which co-regulates virulence with iron concentrations and in which there is a Fur or Fur-like repressor. (Heidrich et al (1996) FEMS 140 253-259, Trivier and Courcol (1996) FEMS 141 117-127).

Briefly and as described above in Example 1, partial diploid analysis in reporter host strains of *Escherichia coli* was performed revealing DtxR(E175K) is dominant in strains which carry DtxR/DtxR(E175K). A recombinant partial diploid [dtxR(E175K) / *SirR*] of *Staphylococcus aureus* was constructed carrying the iron independent E175K mutant DtxR. When this strain was used in a series of mouse challenge experiments, it was found to attenuate virulence in a mouse model of staphylococcal infection [See Figures 9A and 9B] When this strain is compared to the parent strain in a mouse skin lesion model there was a significant decrease in CFU isolated from the lesion after 8 days associated with a significant decrease in lesion size compared to *wt* at each time point through out the study [See Figures 9A

and 9B]. These data further demonstrate that activated E175K DtxR or E175K DtxR/DtxR-like (IdeR or SirR) heterodimeric repressors decrease the virulence of pathogenic microorganisms.

It is noteworthy that a dominant metal ion independent repressor from *C. diphtheriae* suppresses virulence in *S. aureus*, especially since the homology of the two repressors is at the amino acid level approaches only 30% as compared to the nearly 60% identity between IdeR from *M. tuberculosis* and DtxR. This means that dominant metal ion independent repressors can be employed across species barriers to selectively control gene expression to produce desired phenotypical changes.

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5 All publications cited in the specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All these publications are herein incorporated by reference to the same extent as if each individual publication were specifically and individually indicated to be incorporated by reference. In addition, U.S. Provisional Application Nos. 60/161,193, filed October 22, 1999, and 60/161,292, filed October 25, 1999, are hereby incorporated by reference.

10 Although the invention herein has been described with reference to particular embodiments, it is to be understood that these embodiments are merely illustrative of the principles and applications of the present invention. It is therefore to be understood that numerous modifications may be made to the illustrative embodiments and that other arrangements may be devised without departing from the
15 spirit and scope of the present invention as defined by the appended claims.

INDUSTRIAL APPLICABILITY

The present invention is applicable to human and veterinary medicine and more specifically to disease prevention and control of infectious and disease.

CLAIMS

1. A composition comprising a virulent or opportunistic prokaryote in which metal ion-dependent gene regulation confers a growth or an infectious advantage, said prokaryote containing a recombinant DNA molecule comprising a promoter in operable association with a sequence encoding a dominant, metal ion-independent repressor protein or a partially metal ion independent repressor protein, and a carrier.
2. The composition of claim 1 wherein said recombinant DNA molecule is contained in a non-chromosomal vector.
3. The composition of claim 1 wherein said prokaryote is a bacterium.
4. The composition of claim 3 wherein said bacterium is a member of the genus *Mycobacterium*.
5. The composition of claim 4 wherein said bacterium is *Mycobacterium tuberculosis*.
6. The composition of claim 4 wherein said bacterium is *Mycobacterium leprae*.
7. The composition of claim 4 wherein said bacterium is *Mycobacterium avium*.
8. The composition of claim 4 wherein said bacterium is *Mycobacterium paratuberculosis*.
9. The composition of claim 4 wherein said bacterium is *Mycobacterium bovis*.
10. The composition of claim 3 wherein said bacterium is a member of the genus *Staphylococcus*.
11. The composition of claim 10 wherein said bacterium is *Staphylococcus epidermitis*.
12. The composition of claim 10 wherein said bacterium is *Staphylococcus aureus*.

13. The composition of claim 3 wherein said bacterium is a member of the genus *Streptococcus*.

14. The composition of claim 13 wherein said bacterium is *Streptococcus mutans*.

5 15. The composition of claim 13 wherein said bacterium is *Streptococcus pneumoniae*.

16. The composition of claim 1 wherein said sequence encodes a metal ion-independent diphtheria toxin repressor (DtxR) protein.

10 17. The composition of claim 1 wherein said sequence encodes a metal ion-independent or a partially metal ion independent IdeR or SirR repressor protein.

18. The composition of claim 3 wherein said bacterium is a gram-positive bacterium.

15 19. A composition comprising a virulent or opportunistic bacterium in which metal ion-dependent gene regulation confers a growth or an infectious advantage, said bacterium containing a recombinant DNA molecule comprising a promoter in operable association with a sequence encoding a metal or metal ion-independent DtxR protein or a partially metal ion independent DtxR protein, and a carrier.

20 20. An isolated and purified DNA molecule consisting essentially of a sequence encoding a metal ion independent or a partially metal ion independent DtxR or homologue thereof.

21. The DNA molecule of claim 20 wherein said DtxR homolog is IdeR.

25 22. The DNA molecule of claim 20 wherein said DtxR homolog is SirR.

23. A recombinant DNA molecule containing a constitutive promoter element in operable association with the DNA molecule of claim 20.

30 24. A recombinant vector comprising a promoter element in operable association with the DNA molecule of claim 20.

25. The recombinant vector of claim 24 which is a plasmid.

26. A virulent or opportunistic prokaryote in which metal ion-dependent gene regulation confers a growth or an infectious advantage, wherein said prokaryote is transformed with a DNA molecule encoding a dominant, metal ion-independent repressor protein or a partially metal ion-independent repressor protein, and wherein said DNA molecule is expressed in said prokaryote.

27. A method of enhancing protective immunity against infection or disease caused by an opportunistic or virulent prokaryotic pathogen in which metal ion-dependent gene regulation confers a growth or an infectious advantage, comprising administering to an animal the composition of claim 1.

28. The method of claim 27 wherein said animal is a human.

29. The method of claim 27 wherein the prokaryote is in live form.

30. The method of claim 27 wherein the prokaryote is in inactivated form.

31. A method of attenuating or reducing the severity of an infection or disease caused by an opportunistic or virulent prokaryotic pathogen in which metal ion-dependent gene regulation confers a growth or an infectious advantage, comprising administering to an animal the composition of claim 1.

32. The method of claim 1 wherein said animal is a human.

	1	15	16	30	31	45	46	60	61	75	76	90
1 mtblder	MNELVDTTTMYLRAT	YDLEEEGVTPLRARI	AERLDQSGPTVSQT	SRNERDGLRVAGDR	HLELTEKGRALAI	AV	MRKHRLAERLLVDVI					90
2 dtxr	MKDLVDTTTMYLRAT	YDLEEEGVTPLRARI	AERLEQSGPTVSQT	ARNERDGLVVASD	-	SLOMTPTGRTLAT	AV	MRKHRLAERLLTDII				89
3 staphepisirr	-----	-----	MTVSCPPPS--T	SREEQ	-----	ARAL	CLALLTARSRTRAEL	AGQLAKRGCPYEDIGN	RVLDRLA	AVGLVDDT		65
	91	105	106	120	121	135	136	150	151	165	166	180
1 mtblder	GLPWEVHACRW	HVNSDEVRRLLVKVL	NNFTTSPFGNPIPL	VELGVGPEPGADDN	LVR	TELPA	GS	PVAV	VVR	OLTEHVQGDIDL		180
2 dtxr	GLDINKVHACRW	HVNSDEVRRLLVKVK	DVSR-SPFGNPIPL	DELGVGNS--	DA	AP	GTRV	IDA	AT	SPERKV	RIVQ	176
3 staphepisirr	DFAEQVQVSRANAA	KSXRALAEALHAKGV	DDDVITTVLGGIDAG	AERGRAEK--	LVRAR	LRREVLI	DG	TD	DEAR	VSRRLVAM	LARRG-Y	152
	181	195	196	210	211	225	226	240	241	255	256	270
1 mtblder	ITRLKDAGVWPNARV	TVETTPGGGVITVIP	GHEENVTLPIEMAHAV	KVEKV				230				
2 dtxr	FQLLDADIRGVSEVE	IVDRD--GHITLSHN	GKD--VELLDL	LAHITI	RIEEL			223				
3 staphepisirr	GQTLACEVWVIAELAA	ERERR--RV-----	-----	-----	-----	-----	-----	174				

FIGURE 1

2/10

1	1	15	30	45	60	75	90	
1 M1.	MNDLVDTTETNYLRTI	YDLEEGIVTPLRAR	IAER-----	PTVSQT	VSRMERDGLRVAGN	RHLELTGKRAMAIA	VNRKHRLAERLLVDV	85
2 TB	MNELVDTTETNYLRTI	YDLEEG-VTPLRAR	IAERLDQSGPTVSQT	VSRMERDGLRVAGN	RHLELTGKRAMAIA	VNRKHRLAERLLVDV		89
3 M3	MNDLVDTTETNYLRTI	YDLEEG-VTPLRAR	IAERLDQSGPTVSQT	VSRMERDGLRVAGN	RHLELTGKRAMAIA	VNRKHRLAERLLVDV		89
4 M4b	MTVSCPPPTSER--	---EEOARALCLRL	TARS-----	RTRAEL	AGOLAKRGYPEDIGN	RVLID-----	RLAANG LVDDTDFAEQ-----	70
	91	105	120	135	150	165	180	
1 M1.	IGLPMEEVHAEACRM	EHVNSDEVRLIKV	LNNPTSPFGNPIPG	LIDLGAGPDASAANA	KLVRITELPSGSPVA	VVVRQLTEHVND-DID		174
2 TB	IGLPMEEVHAEACRM	EHVNSDEVRLIKV	LNNPTSPFGNPIPG	LVLGVGPEPGADDA	NLVRITELPSGSPVA	VVVRQLTEHVND-DID		179
3 M3	IGLPMEEVHAEACRM	EHVNSDEVRLIKV	LNNPTSPFGNPIPG	LTELAVTFCVNTEDV	SLVRITELPSGSPVA	VVVRQLTEHVND-DID		179
4 M4b	----WVQS-RRANAA	KSKRALAEHLHAKGV	DDDVITTVLGG-IDA	GAERGR--AEKLVRA	RLRREVLIIDGTDEA	RVSRLVAMLA-----		148
	101	195	210	225	240	255	270	
1 M1.	LITRLKDTGVVPNAR	VTVETSPAGNVIIII	PGHENVTLPHEMAHA	VKVEKV----	225			
2 TB	LITRLKDTGVVPNAR	VTVETSPAGNVIIII	PGHENVTLPHEMAHA	VKVEKV----	230			
3 M3	LIGRLKEAGVVPNAR	VTVEANNNGVMIVI	PGIEOVELPHMAHA	VKVEKV----	233			
4 M4b	---RRYGOTLACEV	VIAELAEERERRRV	-----	-----	174			

FIGURE 2

3/10

1	10	20	30	40	50	60
Bl DtxR		MKDLVDTTEM	YLRTIYELEE	EGIVPLRARI	AERLEQSGPT	VSQTVARMER DGLVHVSPDR
Cd DtxR		MKDLVDTTEM	YLRTIYELEE	EGVTPLRARI	AERLEQSGPT	VSQTVARMER DGLVVVASDR
Mt IdeR		MNELVDTTEM	YLRTIYDLEE	EGVTPLRARI	AERLDQSGPT	VSQTVSRMER DGLLRVAGDR
Ms IdeR		MNDLVDTTEM	YLRTIYDLEE	EGVVPLRARI	AERLDQSGPT	VSQTVSRMER DGLLHVAGDR
Ml IdeR		MNDLVDTTEM	YLRTIYDLEE	EGIVTPLRARI	AER PT	VSQTVSRMER DGLLRVAGNR
Sl DesR		MSGLIDTTEM	YLRTILELEE	EGVVPMRARI	AERLDQSGPT	VSQTVARMER DGLVSVAAADR
Se SirR	M	LTEEKED	YLKAILTNDG	DVSFVSNKKL	SQFLNIKPPS	VSEMVGRLK EGYVETKHYK
Sa SirR	M	LTEEKED	YLKAILTNDG	DKNFVTNKIL	SQFLNIKPPS	VSEMVGRLK AGYVETKPYK
Ef	M	TPNKED	YKLIFELGG	DEVKVNKKQI	VSGLDVSAAS	VSEMISKLVK EDLVEHSPYQ
Sg	M	TPNKED	YKCLYELGT	RHNKITNKEI	AGLMQVSPPA	VTEMMKKLLA EELLIKDKKA
Sm	M	TPNKED	YLKIIYELSE	RDEKISNKQI	AEKMSVSAPA	VSEMVKKLLL EDLVLDKQQA
Spn	M	TPNKED	YLKCIYEIGI	DLHKITNKEI	AARMQVSPPA	VTEMIKRMKS ENLILKQKEC
Spy	M	TPNKED	YLKCIYEIGI	DLHKITNKEI	AARMQVSPPA	VTEMIKRMKS ENLILKQKEC

CONSENSUS M---*---E* YL*-I*--- ----NK-* *-----P- ****-*** *-L-----

	70	80	90	100	110	120
Bl DtxR	SLEMTPEGRS	LAIAMVRNDR	LAERLLTDII	GLDIHKVHDE	ACRWEHVMSD	EVERRLVEVL
Cd DtxR	SLQMTPTGRT	LATAVMRKHR	LAERLLTDII	GLDINKVHDE	ACRWEHVMSD	EVERRLVKVL
Mt IdeR	HLELTEKGRA	LAIAMVRKHR	LAERLLVDVI	GLPWEEVHAE	ACRWEHVMSE	DVERRLVKVL
Ms IdeR	HLELTDKGRA	LAVAVMRKHR	LAERLLVDVI	LPWEDGVHAE	ACRWEHVMSE	EVERRLVQVL
Ml IdeR	HLELTTKGRA	MAIAMVRKHR	LAERLLVDVI	GLPWEEVHAE	ACRWEHVMSE	DVERRLIKVL
Sl DesR	HLELTDEGRR	LATRVMRKHR	LAECCLVDVI	GLEWEQVHAE	ACRWEHVMSE	AVERRVLELL
Se SirR	GARLTEEGLK	QTLDIKRRH	LLRLFLIEIL	QYNWEEVHQE	AEILEHRISD	LFVERLDKIL
Sa SirR	GVRLTEDGLT	HTLDII RHR	LLELFLIEIL	KYNWEEVHQE	AEILEHRISD	LFVERLDSLL
Ef	GVQLTEKGLK	KASTLIRKHR	IWEVFLVEHL	NYTWNDVHEE	AEVLEHVTSD	TLVNRLADYL
Sg	GYYLLDLGLK	LVSDLYRKHR	LIEVFLVHHL	GYTTEEIHEE	AEVLEHTVSD	HFVERLDQLL
Sm	GYYLLTKGQI	LASSLYRKHR	LIEVFLMNLH	NYTADEIHEE	AEVLEHTVSD	VFVERLDKFL
Spn	GYYLLDLGLK	LVSELYRKHR	LIEVFLVHHL	DYTSQIHEE	AEVLEHTVSD	LFVERLDKLL
Spy	GYYLLDLGLK	LVSELYRKHR	LIEVFLVHHL	DYTSQIHEE	AEVLEHTVSD	LFVERLDKLL

CONSENSUS ---LT-G-- -----*HR L-*L---L -----H-E A**EH-S* -***R***-L

	130	140	150	160	170	180
Bl DtxR	DDVHRSPFGN	PIPGLEIGL	DQADEPDGSV	RAIDLPLGEN	LKARIVQLNE	ILQVDLEQFQ
Cd DtxR	KDVSRSPPFN	PIPGLELGV	GNSDAAAPGT	RVIDAATSMP	RKVRIVQINE	IFQVETDQFT
Mt IdeR	NNPTTSPFGN	PIPGLELGV	GPEPGADDAN	LVRLTELPAG	SPVAVVVRQL	TEHVQGDIDL
Ms IdeR	ENPTTSPFGN	PIPGLELAV	TPGVNTEDVS	LVRLTELPVG	MPVAVVVRQL	TEHVQGDIDL
Ml IdeR	NNPTTSPFGN	PIPGLLDLGA	GPDASAANAK	LVRLTELPVG	SPVAVVVRQL	TEHV DDIDL
Sl DesR	RHPTESPYGN	PIPGLEELGE	TDGADPFLDE	GMVSLADLDP	GOEGKTVVVR	RIGEPITQDA
Se SirR	NFPKTCPHGG	VIP	R GNSDAAAPGT	SILNFEPGER	VTVRRV RDK	TELL
Sa SirR	NFPKTCPHGG	VIP	R NNEYKEYIT	TILNYEPGDI	VTIKRV RDK	TDLL
Ef	NHPEFCPHGG	VIPEDNQPIH	EEKRQTLTDY	PVGTKIRIAR	VLDEKELLDY	LVSIDLNIQE
Sg	DYPKACPHGG	TIPAKGELLV	EKKHKTLEEA	KEKGDYILAR	VHDNFDLLTY	LERNGLQVGK
Sm	NYPKVCPHGG	TIPGHGQPLV	ERYRTTLKGV	TEMGVYLLKR	VQDNFOLLKY	MEQHHLKIGD
Spn	GFPKTCPHGG	TIPAKGELLV	EINNPLPLADI	KEAGAYRLTR	VHDSFDILHY	LDKHSLLHIGD
Spy	GFPKTCPHGG	TIPAKGELLV	EINNPLPLADI	KEAGAYRLTR	VHDSFDILHY	LDKHSLLHIGD

CONSENSUS --P--*P*G* -IP---*--- ---- ---- ----

FIGURE 3

4/10

	190	200	210	220	230
B1 DtxR	ALTDAGVEIG	TEVDIINEQG	RVVITHNGSS	VELIDDLAHA	VRVEKVEG
Cd DtxR	QLLDADIRVG	SEVEIVDRDG	HITLSHNGKD	VELLDDLAHT	IRIEEL
Mt IdeR	ITRLKDAGVV	PNARVTVETT	PGGGVTVIVIP	GHENVTLPHE	MAHAVKVEKV
Ms IdeR	IGRLKEAGVV	PNARVTVEAN	NNGGVMIVIP	GHEQVELPHH	MAHAVKKKVE KVEKV
M1 IdeR	ITRLKDTGVV	PNARVTVETS	PAGNVIIIP	GHENVTLPHE	MAHAVKVEKV

S1 DesR	QLMYTLRRAG	VQPGSVVSVT	ESAGGVLVGS	GGEAAELEAD	TASHVFAKR
Sa SirR	VYLSSKDIYI	GNTVEIVSKD	DTNKVIILKR	NDIVTILSYE	NAMNIFAEK
Sa SirR	IYLSSKDISI	GNEVEIVSKD	EMNKVIIIKR	NDNVIIVSYE	NAMNMFAEK
Ef	EYTIKEIAAY	EGPITIYNEN	KELSVSFKAA	NTIFVEPLIR	ESEEN
Sg SgoR	TIRFLGYDDF	SHLYSLEVDG	QEIQLAQPIA	QQIYVEKI	
Sm SloR	ELRLLEYDAF	AGAYTIEKDG	EQLQVTSAVA	SQIYIEKKAY	
Spn	QLQVKQFDGF	SNTFTILSND	EDLQVNMDIA	KQLYVEKIN	
Spy	QLQVKQFDGF	SNTFTILSND	EDLQVNMDIA	KQLYVEKIN	

CONSENSUS -----

FIGURE 3 cont.

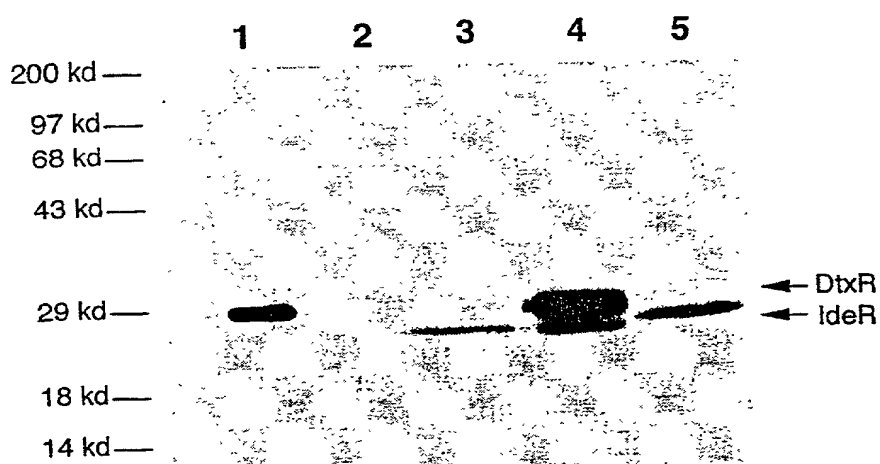


FIGURE 4

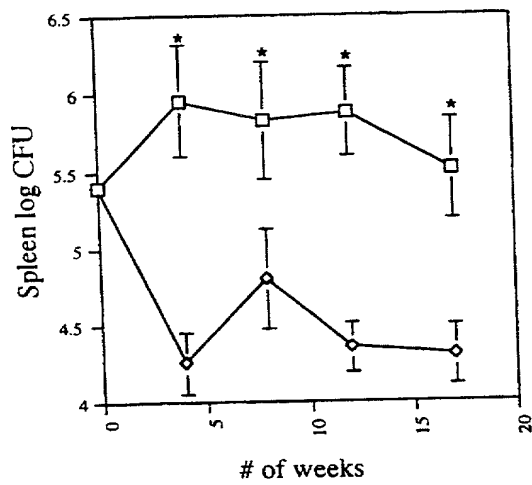


FIGURE 5A

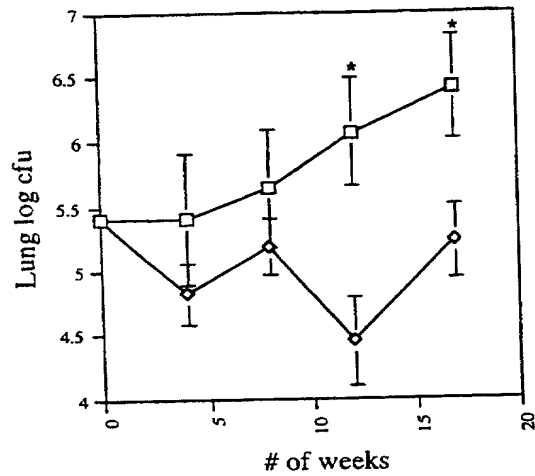


FIGURE 5B

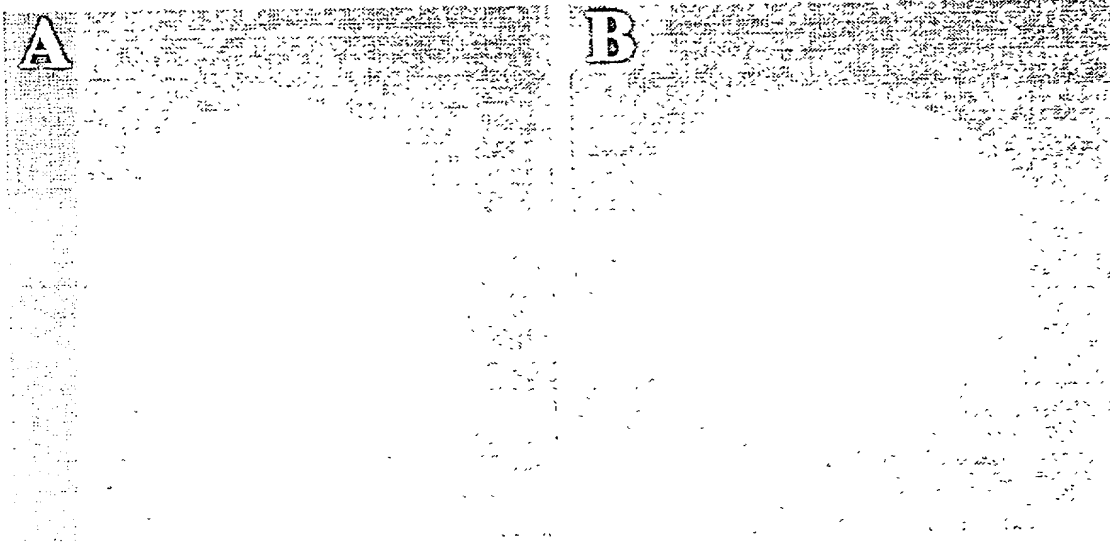


FIGURE 6A

FIGURE 6B

G	T	A	G	G	T	T	A	G	G	C	T	-	A	A	C	C	T	A	T	Consensus Sequence
T	T	A	G	G	T	T	A	G	G	C	T	-	A	A	C	C	T	A	A	Published Consensus
T	T	A	G	G	A	T	A	G	C	T	T	-	T	A	C	C	T	A	A	<i>tox</i> PO <i>C.diphtheriae</i>
T	T	A	G	G	T	T	A	G	G	C	T	-	C	A	C	C	T	A	A	<i>desA</i> PO <i>S.pilosus</i>
C	C	A	G	G	G	T	A	T	C	T	A	-	A	T	C	C	T	G	T	16S ribosomal RNA
G	C	A	G	G	C	C	A	G	T	G	A	-	A	A	C	C	T	G	T	19 kDa antigen
A	C	A	G	G	T	G	G	T	G	C	T	C	A	A	C	C	A	C	G	<i>htrA</i> homologue
G	A	A	G	G	T	A	A	C	G	T	T	C	A	A	C	C	A	A	T	<i>phoP</i> homologue
G	C	A	G	G	T	G	A	C	C	G	T	C	A	A	C	C	G	A	T	<i>adhB</i> homologue
G	A	A	G	G	T	C	A	A	C	C	A	-	A	A	C	A	A	G	A	<i>narG</i> homologue

FIGURE 7

9/10

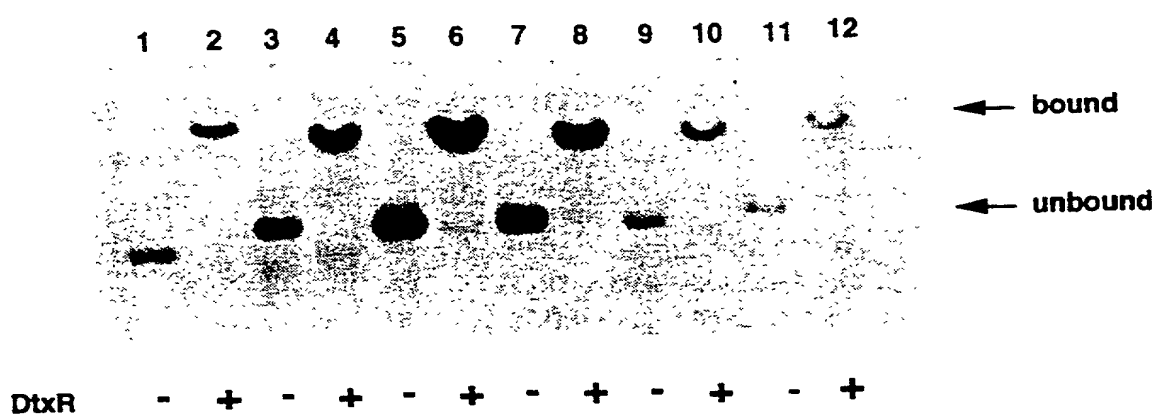


FIGURE 8

10/10

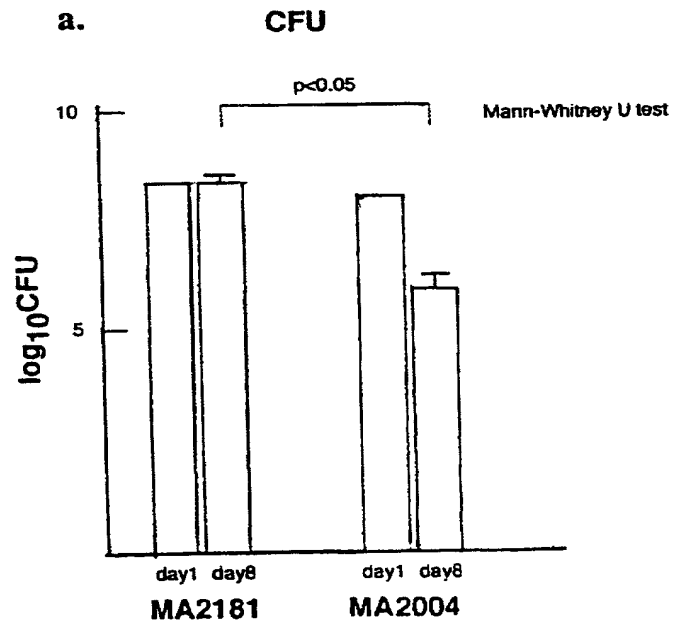


FIGURE 9A

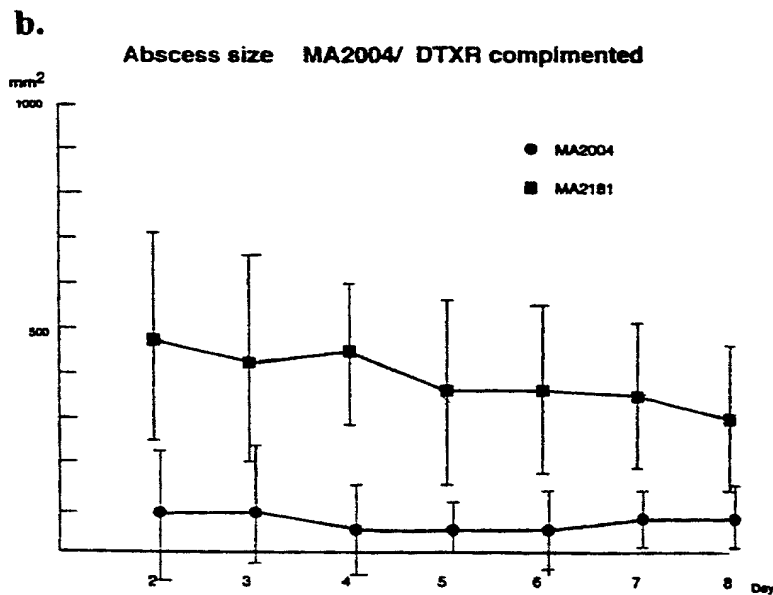


FIGURE 9B

DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION

ATTORNEY'S DOCKET NO.: AMSC 3.3-001

As a below-named inventor, I hereby declare that:

My residence, mailing address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

VACCINE COMPOSITIONS The specification of which

☐ is attached hereto

☒ was filed on October 23, 2000 as United States Application Number or PCT International Application Number PCT/US00/29231 and was amended on (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, § 119(a)-(d) of any foreign application(s) for patent or inventor's certificate or § 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below any foreign application for patent or inventor's certificate, or any PCT international application having a filing date before that of the application on which priority is claimed:

PRIOR FOREIGN APPLICATION(S)			
COUNTRY	APPLICATION NUMBER	DATE OF FILING (month, day, year)	PRIORITY CLAIMED
			YES <input type="checkbox"/> NO <input type="checkbox"/>
			YES <input type="checkbox"/> NO <input type="checkbox"/>
			YES <input type="checkbox"/> NO <input type="checkbox"/>

LISTING OF FOREIGN APPLICATIONS CONTINUED ON PAGE 3 HEREOF ☐ YES ☐ NO

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below:

Application Number: 60/161,193

Filing Date: October 22, 1999 ✓

Application Number: 60/161,292

Filing Date: October 25, 1999 ✓

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s), or § 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

U.S. Parent Application Serial Number:

Parent Filing Date:

Parent Patent No.:

U.S. Parent Application Serial Number:

Parent Filing Date:

Parent Patent No.:

PCT Parent Number:

Parent Filing Date:

LISTING OF US APPLICATIONS CONTINUED ON PAGE 3 HEREOF: ☐ YES ☒ NO

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Customer Number 000530

DIRECT ALL CORRESPONDENCE TO: Customer No. 000530

ATTORNEY DOCKET NO. AMSC 33-001

1-00 Full name of sole or first inventor (given name, family name): John R. MURPHY

Inventor's signature

Residence. Boston, Massachusetts Citizenship US

Mailing Address: 130 Appleton Street, Boston MA 02116, U.S.A.

2-00 Full name of second joint inventor, if any (given name, family name) Edward O'LEARY

Second Inventor's signature

Residence: Wolcott, Connecticut Citizenship: US ✓

Mailing Address: 153 Spindle Hill Road, Wolcott, CT 06716, U.S.A.

2-00 Full name of third joint inventor, if any (given name, family name): Robert J. HARRISON

Third Inventor's signature _____

Residence: Medfield, Massachusetts Citizenship: US

Mailing Address: 31 Green Street, Medfield, MA 02052

Full name of fourth joint inventor, if any (given name, family name):

Fourth Inventor's signature

Residence: Citizenship.

Mailing Address:

Full name of fifth joint inventor (given name, family name):

Fifth Inventor's signature

Residence: Citizenship:

Mailing Address:

Full name of sixth joint inventor, if any (given name, family name):

Sixth Inventor's signature

Residence: Citizenship:

Mailing Address.

Full name of seventh joint inventor, if any (given name, family name):

Seventh Inventor's signature

Residence: Citizenship:

Mailing Address:

Full name of eighth joint inventor, if any (given name, family name):

Eighth Inventor's signature

Residence: Citizenship:

Mailing Address:

☐ Additional inventors are being named on separately numbered sheets attached hereto.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of :
John R. MURPHY et al. :
 :
International Application No. :
PCT/US00/29231 :
 :
International Filing Date: :
23 October 2000 :
 :
For: VACCINE COMPOSITIONS :
 :

X

BOX SEQUENCE
Commissioner for Patents
Washington, D.C. 20231

STATEMENT ACCOMPANYING SEQUENCE LISTING

Sir:

Applicants enclose herewith the sequence listing in computer readable form (*i.e.*, a diskette) as well as a paper copy for the above referenced U.S. National application. The sequence listing does not include matter which goes beyond the content of the Application as filed and the information recorded on the diskette is identical to the written sequence listing.

Respectfully submitted,

LERNER, DAVID, LITTENBERG,
KRUMHOLZ & MENTLIK, LLP


Gina Maldonado

Date: 6/15/01

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SEQUENCE LISTING

<110> Murphy, John R.
O'Lear, Edward
Harrison, Robert J.

<120> Vaccine Compositions

<130> AMSC 3.3-001

<140> To be assigned

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<150> PCT/US00/29231

<151> 2000-10-23

<160> 36

<170> PatentIn Ver. 2.1

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operator

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 Consensus-binding sequence

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 <223> "n" represents variable bases

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 Asp Leu Glu Glu Glu Gly Val Thr Pro Leu Arg Ala Arg Ile Ala Glu
 20 25 30
 Arg Leu Asp Gln Ser Gly Pro Thr Val Ser Gln Thr Val Ser Arg Met
 35 40 45

Glu Arg Asp Gly Leu Leu Arg Val Ala Gly Asp Arg His Leu Glu Leu
 50 55 60
 Thr Glu Lys Gly Arg Ala Leu Ala Ile Ala Val Met Arg Lys His Arg
 65 70 75 80
 Leu Ala Glu Arg Leu Leu Val Asp Val Ile Gly Leu Pro Trp Glu Glu
 85 90 95
 Val His Ala Glu Ala Cys Arg Trp Glu His Val Asn Ser Glu Asp Val
 100 105 110
 Glu Arg Arg Leu Val Lys Val Leu Asn Asn Pro Thr Thr Ser Pro Phe
 115 120 125
 Gly Asn Pro Ile Pro Gly Leu Val Glu Leu Gly Val Gly Pro Glu Pro
 130 135 140
 Gly Ala Asp Asp Ala Asn Leu Val Arg Leu Thr Glu Leu Pro Ala Gly
 145 150 155 160
 Ser Pro Val Ala Val Val Val Arg Gln Leu Thr Glu His Val Gln Gly
 165 170 175
 Asp Ile Asp Leu Ile Thr Arg Leu Lys Asp Ala Gly Val Val Pro Asn
 180 185 190
 Ala Arg Val Thr Val Glu Thr Thr Pro Gly Gly Gly Val Thr Ile Val
 195 200 205
 Ile Pro Gly His Glu Asn Val Thr Leu Pro His Glu Met Ala His Ala
 210 215 220
 Val Lys Val Glu Lys Val
 225 230

<210> 8
 <211> 223
 <212> PRT
 <213> *Corynebacterium diphtheriae*

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 1 5 10 15
 Glu Leu Glu Glu Glu Gly Val Thr Pro Leu Arg Ala Arg Ile Ala Glu
 20 25 30
 Arg Leu Glu Gln Ser Gly Pro Thr Val Ser Gln Thr Val Ala Arg Met
 35 40 45
 Glu Arg Asp Gly Leu Val Val Val Ala Ser Asp Ser Leu Gln Met Thr
 50 55 60
 Pro Thr Gly Arg Thr Leu Ala Thr Ala Val Met Arg Lys His Arg Leu
 65 70 75 80

<210> 12
 <211> 233
 <212> PRT
 <213> Mycobacterium smegmatis

<400> 12

Met	Asn	Asp	Leu	Val	Asp	Thr	Thr	Glu	Asn	Tyr	Leu	Arg	Thr	Ile	Tyr
1				5					10					15	
Asp	Leu	Glu	Glu	Glu	Gly	Val	Val	Pro	Leu	Arg	Ala	Arg	Ile	Ala	Glu
		20						25					30		
Arg	Leu	Asp	Gln	Ser	Gly	Pro	Thr	Val	Ser	Gln	Thr	Val	Ser	Arg	Met
		35					40					45			
Glu	Arg	Asp	Gly	Leu	Leu	His	Val	Ala	Gly	Asp	Arg	His	Leu	Glu	Leu
	50					55					60				
Thr	Asp	Lys	Gly	Arg	Ala	Leu	Ala	Val	Ala	Val	Met	Arg	Lys	His	Arg
	65				70					75					80
Leu	Ala	Glu	Arg	Leu	Leu	Val	Asp	Val	Ile	Gly	Leu	Pro	Trp	Glu	Asp
				85					90					95	
Val	His	Ala	Glu	Ala	Cys	Arg	Trp	Glu	His	Val	Met	Ser	Glu	Glu	Val
			100					105					110		
Glu	Arg	Arg	Leu	Val	Gln	Val	Leu	Glu	Asn	Pro	Thr	Thr	Ser	Pro	Phe
		115					120						125		
Gly	Asn	Pro	Ile	Pro	Gly	Leu	Thr	Glu	Leu	Ala	Val	Thr	Pro	Gly	Val
	130					135					140				
Asn	Thr	Glu	Asp	Val	Ser	Leu	Val	Arg	Leu	Thr	Glu	Leu	Pro	Val	Gly
	145				150					155					160
Met	Pro	Val	Ala	Val	Val	Val	Arg	Gln	Leu	Thr	Glu	His	Val	Gln	Gly
			165						170					175	
Asp	Thr	Asp	Leu	Ile	Gly	Arg	Leu	Lys	Glu	Ala	Gly	Val	Val	Pro	Asn
			180					185					190		
Ala	Arg	Val	Thr	Val	Glu	Ala	Asn	Asn	Asn	Gly	Gly	Val	Met	Ile	Val
		195					200					205			
Ile	Pro	Gly	His	Glu	Gln	Val	Glu	Leu	Pro	His	His	Met	Ala	His	Ala
	210					215					220				
Val	Lys	Val	Glu	Lys	Val	Glu	Lys	Val							
	225				230										

<210> 13
 <211> 174
 <212> PRT

<213> Mycobacterium tuberculosis

<400> 13

Met Thr Val Ser Cys Pro Pro Pro Ser Thr Ser Glu Arg Glu Glu Gln
1 5 10 15

Ala Arg Ala Leu Cys Leu Arg Leu Leu Thr Ala Arg Ser Arg Thr Arg
20 25 30

Ala Glu Leu Ala Gly Gln Leu Ala Lys Arg Gly Tyr Pro Glu Asp Ile
35 40 45

Gly Asn Arg Val Leu Asp Arg Leu Ala Ala Val Gly Leu Val Asp Asp
50 55 60

Thr Asp Phe Ala Glu Gln Trp Val Gln Ser Arg Arg Ala Asn Ala Ala
65 70 75 80

Lys Ser Lys Arg Ala Leu Ala Ala Glu Leu His Ala Lys Gly Val Asp
85 90 95

Asp Asp Val Ile Thr Thr Val Leu Gly Gly Ile Asp Ala Gly Ala Glu
100 105 110

Arg Gly Arg Ala Glu Lys Leu Val Arg Ala Arg Leu Arg Arg Glu Val
115 120 125

Leu Ile Asp Asp Gly Thr Asp Glu Ala Arg Val Ser Arg Arg Leu Val
130 135 140

Ala Met Leu Ala Arg Arg Gly Tyr Gly Gln Thr Leu Ala Cys Glu Val
145 150 155 160

Val Ile Ala Glu Leu Ala Ala Glu Arg Glu Arg Arg Arg Val
165 170

<210> 14

<211> 228

<212> PRT

<213> Brevibacterium lactofermentum

<400> 14

Met Lys Asp Leu Val Asp Thr Thr Glu Met Tyr Leu Arg Thr Ile Tyr
1 5 10 15

Glu Leu Glu Glu Glu Gly Ile Val Pro Leu Arg Ala Arg Ile Ala Glu
20 25 30

Arg Leu Glu Gln Ser Gly Pro Thr Val Ser Gln Thr Val Ala Arg Met
35 40 45

Glu Arg Asp Gly Leu Val His Val Ser Pro Asp Arg Ser Leu Glu Met
50 55 60

Thr Pro Glu Gly Arg Ser Leu Ala Ile Ala Val Met Arg Asn Asp Arg
65 70 75 80

Gly Asn Pro Ile Pro Gly Leu Val Glu Leu Gly Val Gly Pro Glu Pro
 130 135 140

Gly Ala Asp Asp Ala Asn Leu Val Arg Leu Thr Glu Leu Pro Ala Gly
 145 150 155 160

Ser Pro Val Ala Val Val Val Arg Gln Leu Thr Glu His Val Gln Gly
 165 170 175

Asp Ile Asp Leu Ile Thr Arg Leu Lys Asp Ala Gly Val Val Pro Asn
 180 185 190

Ala Arg Val Thr Val Glu Thr Thr Pro Gly Gly Gly Val Thr Ile Val
 195 200 205

Ile Pro Gly His Glu Asn Val Thr Leu Pro His Glu Met Ala His Ala
 210 215 220

Val Lys Val Glu Lys Val
 225 230

<210> 17

<211> 235

<212> PRT

<213> Mycobacterium smegmatis

<400> 17

Met Asn Asp Leu Val Asp Thr Thr Glu Met Tyr Leu Arg Thr Ile Tyr
 1 5 10 15

Asp Leu Glu Glu Glu Gly Val Val Pro Leu Arg Ala Arg Ile Ala Glu
 20 25 30

Arg Leu Asp Gln Ser Gly Pro Thr Val Ser Gln Thr Val Ser Arg Met
 35 40 45

Glu Arg Asp Gly Leu Leu His Val Ala Gly Asp Arg His Leu Glu Leu
 50 55 60

Thr Asp Lys Gly Arg Ala Leu Ala Val Ala Val Met Arg Lys His Arg
 65 70 75 80

Leu Ala Glu Arg Leu Leu Val Asp Val Ile Leu Pro Trp Glu Asp Gly
 85 90 95

Val His Ala Glu Ala Cys Arg Trp Glu His Val Met Ser Glu Glu Val
 100 105 110

Glu Arg Arg Leu Val Gln Val Leu Glu Asn Pro Thr Thr Ser Pro Phe
 115 120 125

Gly Asn Pro Ile Pro Gly Leu Thr Glu Leu Ala Val Thr Pro Gly Val
 130 135 140

Asn Thr Glu Asp Val Ser Leu Val Arg Leu Thr Glu Leu Pro Val Gly
 145 150 155 160

Met Pro Val Ala Val Val Val Arg Gln Leu Thr Glu His Val Gln Gly
165 170 175

Asp Thr Asp Leu Ile Gly Arg Leu Lys Glu Ala Gly Val Val Pro Asn
180 185 190

Ala Arg Val Thr Val Glu Ala Asn Asn Asn Gly Gly Val Met Ile Val
195 200 205

Ile Pro Gly His Glu Gln Val Glu Leu Pro His His Met Ala His Ala
210 215 220

Val Lys Lys Lys Val Glu Lys Val Glu Lys Val
225 230 235

<210> 18
<211> 225
<212> PRT
<213> Mycobacterium leprae

<400> 18
Met Asn Asp Leu Val Asp Thr Thr Glu Met Tyr Leu Arg Thr Ile Tyr
1 5 10 15

Asp Leu Glu Glu Glu Gly Ile Val Thr Pro Leu Arg Ala Arg Ile Ala
20 25 30

Glu Arg Pro Thr Val Ser Gln Thr Val Ser Arg Met Glu Arg Asp Gly
35 40 45

Leu Leu Arg Val Ala Gly Asn Arg His Leu Glu Leu Thr Thr Lys Gly
50 55 60

Arg Ala Met Ala Ile Ala Val Met Arg Lys His Arg Leu Ala Glu Arg
65 70 75 80

Leu Leu Val Asp Val Ile Gly Leu Pro Trp Glu Glu Val His Ala Glu
85 90 95

Ala Cys Arg Trp Glu His Val Met Ser Glu Asp Val Glu Arg Arg Leu
100 105 110

Ile Lys Val Leu Asn Asn Pro Thr Thr Ser Pro Phe Gly Asn Pro Ile
115 120 125

Pro Gly Leu Leu Asp Leu Gly Ala Gly Pro Asp Ala Ser Ala Ala Asn
130 135 140

Ala Lys Leu Val Arg Leu Thr Glu Leu Pro Ser Gly Ser Pro Val Ala
145 150 155 160

Val Val Val Arg Gln Leu Thr Glu His Val Asp Asp Ile Asp Leu Ile
165 170 175

Thr Arg Leu Lys Asp Thr Gly Val Val Pro Asn Ala Arg Val Thr Val

09000733 064304

180	185	190
Glu Thr Ser Pro Ala Gly Asn Val Ile Ile Ile Ile Pro Gly His Glu		
195	200	205
Asn Val Thr Leu Pro His Glu Met Ala His Ala Val Lys Val Glu Lys		
210	215	220
Val		
225		
<210> 19		
<211> 230		
<212> PRT		
<213> Streptomyces lividans		
<400> 19		
Met Ser Gly Leu Ile Asp Thr Thr Glu Met Tyr Leu Arg Thr Ile Leu		
1	5	10 15
Glu Leu Glu Glu Glu Gly Val Val Pro Met Arg Ala Arg Ile Ala Glu		
20	25	30
Arg Leu Asp Gln Ser Gly Pro Thr Val Ser Gln Thr Val Ala Arg Met		
35	40	45
Glu Arg Asp Gly Leu Val Ser Val Ala Ala Asp Arg His Leu Glu Leu		
50	55	60
Thr Asp Glu Gly Arg Arg Leu Ala Thr Arg Val Met Arg Lys His Arg		
65	70	75 80
Leu Ala Glu Cys Leu Leu Val Asp Val Ile Gly Leu Glu Trp Glu Gln		
85	90	95
Val His Ala Glu Ala Cys Arg Trp Glu His Val Met Ser Glu Ala Val		
100	105	110
Glu Arg Arg Val Leu Glu Leu Leu Arg His Pro Thr Glu Ser Pro Tyr		
115	120	125
Gly Asn Pro Ile Pro Gly Leu Glu Glu Leu Gly Glu Thr Asp Gly Ala		
130	135	140
Asp Pro Phe Leu Asp Glu Gly Met Val Ser Leu Ala Asp Leu Asp Pro		
145	150	155 160
Gly Gln Glu Gly Lys Thr Val Val Val Arg Arg Ile Gly Glu Pro Ile		
165	170	175
Gln Thr Asp Ala Gln Leu Met Tyr Thr Leu Arg Arg Ala Gly Val Gln		
180	185	190
Pro Gly Ser Val Val Ser Val Thr Glu Ser Ala Gly Gly Val Leu Val		
195	200	205

Gly Ser Gly Gly Glu Ala Ala Glu Leu Glu Ala Asp Thr Ala Ser His
 210 215 220

Val Phe Val Ala Lys Arg
 225 230

<210> 20
 <211> 215
 <212> PRT
 <213> Staphylococcus epidermidis

<400> 20
 Met Leu Thr Glu Glu Lys Glu Asp Tyr Leu Lys Ala Ile Leu Thr Asn
 1 5 10 15
 Asp Gly Asp Val Ser Phe Val Ser Asn Lys Lys Leu Ser Gln Phe Leu
 20 25 30
 Asn Ile Lys Pro Pro Ser Val Ser Glu Met Val Gly Arg Leu Glu Lys
 35 40 45
 Glu Gly Tyr Val Glu Thr Lys His Tyr Lys Gly Ala Arg Leu Thr Glu
 50 55 60
 Glu Gly Leu Lys Gln Thr Leu Asp Ile Ile Lys Arg His Arg Leu Leu
 65 70 75 80
 Arg Leu Phe Leu Ile Glu Ile Leu Gln Tyr Asn Trp Glu Glu Val His
 85 90 95
 Gln Glu Ala Glu Ile Leu Glu His Arg Ile Ser Asp Leu Phe Val Glu
 100 105 110
 Arg Leu Asp Lys Ile Leu Asn Phe Pro Lys Thr Cys Pro His Gly Gly
 115 120 125
 Val Ile Pro Arg Gly Asn Ser Asp Ala Ala Ala Pro Gly Thr Ser Ile
 130 135 140
 Leu Asn Phe Glu Pro Gly Glu Arg Val Thr Val Arg Arg Val Arg Arg
 145 150 155 160
 Asp Lys Thr Glu Leu Leu Val Tyr Leu Ser Ser Lys Asp Ile Tyr Ile
 165 170 175
 Gly Asn Thr Val Glu Ile Val Ser Lys Asp Asp Thr Asn Lys Val Ile
 180 185 190
 Ile Leu Lys Arg Asn Asp Ile Val Thr Ile Leu Ser Tyr Glu Asn Ala
 195 200 205
 Met Asn Ile Phe Ala Glu Lys
 210 215

<210> 21

<211> 213
 <212> PRT
 <213> Staphylococcus aureus

<400> 21

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Met Leu Thr Glu Glu Lys Glu Asp Tyr Leu Lys Ala Ile Leu Thr Asn
  1              5              10              15

Asn Gly Asp Lys Asn Phe Val Thr Asn Lys Ile Leu Ser Gln Phe Leu
      20              25              30

Asn Ile Lys Pro Pro Ser Val Ser Glu Met Val Gly Arg Leu Glu Lys
      35              40              45

Ala Gly Tyr Val Glu Thr Lys Pro Tyr Lys Gly Val Arg Leu Thr Glu
      50              55              60

Asp Gly Leu Thr His Thr Leu Asp Ile Ile Arg His Arg Leu Leu Glu
      65              70              75              80

Leu Phe Leu Ile Glu Ile Leu Lys Tyr Asn Trp Glu Glu Val His Gln
      85              90              95

Glu Ala Glu Ile Leu Glu His Arg Ile Ser Asp Leu Phe Val Glu Arg
      100              105              110

Leu Asp Ser Leu Leu Asn Phe Pro Glu Thr Cys Pro His Gly Gly Val
      115              120              125

Ile Pro Arg Asn Asn Glu Tyr Lys Glu Lys Tyr Ile Thr Thr Ile Leu
      130              135              140

Asn Tyr Glu Pro Gly Asp Ile Val Thr Ile Lys Arg Val Arg Asp Lys
      145              150              155              160

Thr Asp Leu Leu Ile Tyr Leu Ser Ser Lys Asp Ile Ser Ile Gly Asn
      165              170              175

Glu Val Glu Ile Val Ser Lys Asp Glu Met Asn Lys Val Ile Ile Ile
      180              185              190

Lys Arg Asn Asp Asn Val Ile Ile Val Ser Tyr Glu Asn Ala Met Asn
      195              200              205

Met Phe Ala Glu Lys
      210
  
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<210> 22
 <211> 222
 <212> PRT
 <213> Enterococcus faecalis

<400> 22

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Met Thr Pro Asn Arg Glu Asp Tyr Leu Lys Leu Ile Phe Glu Leu Gly
  1              5              10              15
  
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Ala Asp Ile Lys Glu Ala Gly Ala Tyr Arg Leu Thr Arg Val His Asp
145 150 155 160

Ser Phe Asp Ile Leu His Tyr Leu Asp Lys His Ser Leu His Ile Gly
165 170 175

Asp Gln Leu Gln Val Lys Gln Phe Asp Gly Phe Ser Asn Thr Phe Thr
180 185 190

Ile Leu Ser Asn Asp Glu Asp Leu Gln Val Asn Met Asp Ile Ala Lys
195 200 205

Gln Leu Tyr Val Glu Lys Ile Asn
210 215

<210> 26

<211> 216

<212> PRT

<213> Streptococcus pyogenes

<400> 26

Met Thr Pro Asn Lys Glu Asp Tyr Leu Lys Cys Ile Tyr Glu Ile Gly
1 5 10 15

Ile Asp Leu His Lys Ile Thr Asn Lys Glu Ile Ala Ala Arg Met Gln
20 25 30

Val Ser Pro Pro Ala Val Thr Glu Met Ile Lys Arg Met Lys Ser Glu
35 40 45

Asn Leu Ile Leu Lys Asp Lys Glu Cys Gly Tyr Leu Leu Thr Asp Leu
50 55 60

Gly Leu Lys Leu Val Ser Glu Leu Tyr Arg Lys His Arg Leu Ile Glu
65 70 75 80

Val Phe Leu Val His His Leu Asp Tyr Thr Ser Asp Gln Ile His Glu
85 90 95

Glu Ala Glu Val Leu Glu His Thr Val Ser Asp Leu Phe Val Glu Arg
100 105 110

Leu Asp Lys Leu Leu Gly Phe Pro Lys Thr Cys Pro His Gly Gly Thr
115 120 125

Ile Pro Ala Lys Gly Glu Leu Leu Val Glu Ile Asn Asn Leu Pro Leu
130 135 140

Ala Asp Ile Lys Glu Ala Gly Ala Tyr Arg Leu Thr Arg Val His Asp
145 150 155 160

Ser Phe Asp Ile Leu His Tyr Leu Asp Lys His Ser Leu His Ile Gly
165 170 175

Asp Gln Leu Gln Val Lys Gln Phe Asp Gly Phe Ser Asn Thr Phe Thr
180 185 190

Ile Leu Ser Asn Asp Glu Asp Leu Gln Val Asn Met Asp Ile Ala Lys
 195 200 205

Gln Leu Tyr Val Glu Lys Ile Asn
 210 215

<210> 27

<211> 19

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Consensus
 sequence

<400> 27

gtaggttagg ctaacctat

19

<210> 28

<211> 19

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Consensus
 sequence

<400> 28

ttaggttagg ctaacctaa

19

<210> 29

<211> 19

<212> DNA

<213> *Cornyebacterium diptheriae*

<400> 29

ttaggatagc ttacctaa

19

<210> 30

<211> 19

<212> DNA

<213> *Streptomyces pilosus*

<400> 30

ttaggttagg ctcacctaa

19

<210> 31

<211> 19

<212> DNA

<213> Unknown Organism

<220>
 <223> Description of Unknown Organism: 16S ribosomal RNA

 <400> 31
 ccagggtatc taatcctgt 19

 <210> 32
 <211> 19
 <212> DNA
 <213> Unknown Organism

 <220>
 <223> Description of Unknown Organism: 19 kDa antigen

 <400> 32
 gcaggccagt gaaacctgt 19

 <210> 33
 <211> 20
 <212> DNA
 <213> Unknown Organism

 <220>
 <223> Description of Unknown Organism: htrA homologue

 <400> 33
 acaggtggtg ctcaaccacg 20

 <210> 34
 <211> 20
 <212> DNA
 <213> Unknown Organism

 <220>
 <223> Description of Unknown Organism: phoP homologue

 <400> 34
 gaaggtaacg ttcaaccaat 20

 <210> 35
 <211> 20
 <212> DNA
 <213> Unknown Organism

 <220>
 <223> Description of Unknown Organism: adhB homologue

 <400> 35
 gcaggtgacc gtcaaccgat 20

 <210> 36
 <211> 19

<213> Unknown Organism

 $\langle 220 \rangle$

<223> Description of Unknown Organism: narG homologue

<400> 36

gaagggtcaac caaacaaga

19